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CHARACTERIZATION OF THE INSECTICIDAL CRYSTAL PROTEIN FROM *BACILLUS THURINGIENSIS*

by

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Thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Master's of Science in Biochemistry.

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CANADA K1N 6N5

ISBN 0-315-60089-6

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENT

The work presented in this thesis could never have been accomplished without the help and support of many friends and colleagues.

I would like to express my gratitude to Drs. Paul Carey and Harvey Kaplan for giving me the opportunity of becoming a graduate student. Special thanks go to Dr. Kaplan for his guidance, his encouragement as well as for sharing his extensive knowledge in this field.

I would like to thank Dr. George Oda for being there to answer all the technical questions and for his help in setting ailing experiments straight.

The excellent technical assistance of Mr. H. Lassarus is gratefully acknowledged and finally, I would like to thank Mrs. Tina Raynor for her help in the preparation of this manuscript.

DEDICATION

TO MY FAMILY

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ABSTRACT

Bacillus thuringiensis produces a crystalline inclusion body composed of a 130-kDa protein which is rendered toxic upon ingestion by lepidoteran larvae. It was shown that proteinases adsorb on the surface of the crystalline body lead to proteolysis of the protein crystal especially on solubilization in alkali. Extensive washing of the protein crystal was shown to remove these proteinases and give a stable preparation. Exposure of the protein crystal to simulated sunlight results in a loss of toxicity and in the destruction of the side-chains of tryptophan, histidine, tyrosine and methionine. After 32 h of irradiation, the amount of destruction reaches a plateau at a loss of 35% tryptophan, 20% histidine, 5% tyrosine and 13% methionine. The rate at which toxicity is lost is much greater than the rate of destruction of these side-chains. It is therefore concluded that destruction of amino acid side-chains is not the primary cause of the photo-inactivation of the protein crystal. The protein in the crystals from the subspecies *kurstaki* HD-1 and *entomocidus* was found to contain 16-18 cysteine residues per molecule, present primarily in the disulfide form as cystine. Evidence that all the cysteine residues form symmetrical

interchain disulfide linkages in the protein crystal was obtained from the following results: (i) The disulfide diagonal procedure (Brown & Hartley, Biochem. J. **101**, 214-228, 1966) gave only unpaired cysteic acid peptides in diagonal maps; (ii) the disulfide bridges were shown to be labile in dilute alkali and the crystal protein could be released quantitatively with 1mM 2-mercaptoethanol; (iii) the sulfhydryl groups of the released crystal protein were shown by competitive labelling (Kaplan et al. Biochem. J. **124**, 289-299, 1971) to have the same chemical properties as exposed groups on the surface of the protein (iv) the sulfhydryl groups in the released crystal protein could be reacted quantitatively with iodoacetate or iodoacetamide. The finding that all the disulfide linkages in the protein crystal are interchain and symmetrical accounts for its alkaline lability and for the high degree of conservation in the primary structure of the cystine-containing regions of the protein from various subspecies.

INTRODUCTION

Overview

The widespread use of insecticides for agricultural and forestry purposes has awakened concerns about the dumping of manufactured chemicals into the ecosystem. Concerns about DDT and other chemical products have created a demand for a safer and more natural method of pest control, which bioinsecticides appear to fill. The fundamental advantage of bioinsecticides over their chemical counterparts is that there is a very narrow spectrum of organisms for which they are toxic. This limited target specificity gives rise to the possibility of tailoring insecticidal formulations for specific pests while leaving other organisms undisturbed.

There exists a varied and diverse group of microorganisms which are pathogenic for insects; included in this group are viruses , bacteria and fungi. Bacterial insecticides are comprised of a broad range of compounds that vary in their mode of action and mechanism of pathogenicity.

Bacillus thuringiensis formulations are currently used as bioinsecticides against pests in the forestry industry as well as in agriculture. Within the last 10 years B. thuringiensis has been recognized as a prime candidate for the production of improved insecticidal toxins through genetic

engineering. Understanding the factors which affect the efficacy of B. thuringiensis requires knowledge of the structure of the insecticidal element as well as an understanding of the mechanism of action. This knowledge will aid in the development of an improved insecticide.

Bacillus thuringiensis is a gram positive soil bacterium distinguishable from the closely related species Bacillus cereus and Bacillus anthracis by the large insecticidal parasporal inclusion that appears during sporulation. This crystal is known as the (δ) delta-endotoxin, to distinguish it from the other toxins produced by this organism. Unlike other sporulation-dependent proteins, the crystal protein is accumulated in large amounts, as a crystalline inclusion body.

By 1987, twenty-eight subspecies of B. thuringiensis had been identified (Himeno, M., 1987). Several methods have been used to classify strains of B. thuringiensis into serotypes, subspecies (subsp.) or varieties (var.). The most commonly used classification system is based on flagella antigens (Himeno, M., 1987). Further classification is achieved by comparing the biochemical differences of these subspecies within a serotype. The different subspecies are toxic to the larvae of many different lepidoptera; each subspecies, and sometimes individual strains within the same subspecies, have

a characteristic spectrum of insect toxicities (Dulmage, H.T., 1978). This classification scheme suffers some shortcomings in that isolates which are classified as being identical show very different toxicities. Ellar, D., Thomas, W., Knowels, B., Ward, E., Todd, J., Drobniewski, F., Lewis, J., Sawyer, T., Last, D. and Nichols, C. (1985) proposed a new classification scheme based on the order of insects to which the isolate is toxic. This system separates the different subspecies into (1) lepidopteran specific (i.e. moths and butterflies) (2) dipteran specific (black flies and mosquitoes) (3) coleopteran specific (beetles) (4) lepidopteran and dipteran specific and (5) no known insecticidal activity.

History of B. thuringiensis

The organism was first isolated in 1901 by Ishiwata (Ishiwata, S., 1901) from diseased silkworm larvae and was subsequently named Bacillus sotto or, in the current nomenclature, Bacillus thuringiensis subsp. sotto. The name Bacillus thuringiensis dates from 1911 when Berliner isolated a crystal-containing organism from diseased flour moth larvae. Little interest was shown in B. thuringiensis until 1954, when Angus showed that the crystalline inclusion body was responsible for the insecticidal action (Angus, T. 1954).

Angus (1956) also showed that the crystal was made up of protein (Angus, T. 1956).

Steinhous (1951) demonstrated that B. thuringiensis could reduce the population of alfalfa caterpillars and based on this work, the first commercial preparation of B. thuringiensis was available for testing by entomologists by 1958 (Hall, I., 1963 and Heimpel, A. and Angus, T. 1963). The first standard for B. thuringiensis toxicity was developed in France in 1965. The more widely used standard, developed in the United States, was developed in 1973 and is based on the "spore-crystal" complex of B. thuringiensis subsp. kurstaki HD-1-S-1971 (Reichelderfer, C., 1985). The widespread use of B. thuringiensis did not start until the early 1980's (Rusco, C., 1987), at which time it was also deployed in Canada.

To date, B. thuringiensis is used in about half of all spruce budworm control operations in Eastern Canada and also for the control of jackpine budworms in Ontario and Manitoba, Western spruce budworm in British Columbia, hemlock looper in Newfoundland and gypsy moth in Ontario (Statistics Canada, 1987).

Molecular genetics of B. thuringiensis

Data obtained using a wide range of techniques indicate that the crystal protein genes in many subspecies of B.

thuringiensis are located on one or more large plasmids. Some genes have also been reported to be located on chromosomal DNA (Aronson, A., Beckman, W. and Dunn, P., 1986 and Brousseau, R., and Masson, L., 1988). The most complete survey of the plasmid content of B. thuringiensis strains currently available is that of Carleton, C. and Gonzale, Z., 1985. Their study of twenty-one subspecies revealed that the number of plasmids varied from 2 to 12 per strain with size ranging from 1.5 to 150 MDa. Since this thesis will only deal with two lepidopteran specific subspecies, B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus as well as with the commercial formulation thuricide (an HD-1 formulation) (Sandoz, Bazel Switzerland), the scope of the review of the gene will be limited to these two subspecies.

When the total DNA from B. thuringiensis subsp. kurstaki HD-1 was digested with Hind III restriction enzyme, run on an agarose gel and blotted on nitrocellulose (southern blot), three bands were found to hybridize with a DNA probe corresponding to the 5' end of the toxic gene. The sizes of these fragments were 4.5, 5.3 and 6.6 kilobase, and therefore the three genes corresponding to these fragments are refered to as the "4.5", "5.3" and "6.6" kb-genes (Kronstad, J. and Whiteley, H., 1986). Since this technique utilizes a specific

probe, it is entirely possible that various B. thuringiensis strains contain different genes whose 5' end is not homologous to the probe used above.

Furthermore, variation in other regions of the gene will not be distinguished by this classification. As such, the genes which hybridize to one of these 4.5 kb probes are refered to as "4.5" kb-gene type. The three gene types mentioned above have been isolated and sequenced.

The "4.5" gene type

This gene was first isolated from B. thuringiensis subsp. kurstaki HD-1 and sequenced by Schneph, H., Wong, H. and Whiteley, H., 1985. The gene coded for a protein of 1176 amino acids with a predicted molecular mass of 133.5-kDa. A second sequence published for the "4.5" kb-genes proved to be almost identical. Kondo, S., Tamura, W., Kuwitate, A., Hattori, M., Akashi, A. and Ohmori, I., (1987) published a sequence of a "4.5" kb-gene also isolated from B. thuringiensis subsp. kurstaki HD-1. Their sequence only differs from that of Schneph et al., (1985) by one base. A partial sequence isolated from B. thuringiensis subsp. sotto was pusblished by Shibano, Y., Yamugata, A., Nakamura, N., Iizuka, T., Sugisaki, H. and Takanami, M. (1985). The first 926 codons published showed a similarity of 99.2% with the two other gene sequences

published.

The "5.3" gene type

There are at present four published sequences for the "5.3" kb-gene type isolated from B. thuringiensis subsp. kurstaki HD-1. These sequences vary by one or two base pairs at most in the whole coding region, which codes for a protein of 1155 amino acids (Brousseau, R. and Masson, L. 1988). Four more sequences isolated from different subspecies are also published and have virtually 100% homology (Brousseau, R. and Masson, L. 1988). Since these sequences were isolated from subspecies which have very different toxicities towards specific insect pests, it raises questions about the serotyping classification scheme.

Kondo, S. et al., 1987 demonstrated a significant toxicity difference between the products of the "4.5" and "5.3" kb-gene products expressed in E. coli. In direct assays against Bombyx mori 3rd instar larvae, the "4.5" kb-gene product was shown to be approximately 10 times more active in terms of mortality after 72 hrs.

The "6.6" gene type

Adang, M., Staver, M., Rocheleau, T., Leighton, J., Barker, R. and Thompson, D. (1985) have published the only "6.6" kb-gene sequence to date. This gene codes for a protein

of 1179 amino acids.

Based on the amount of homology seen among the three gene "types", the protein can be divided into four regions. The first region consists of the N-terminal 282 amino acids which are 98% homologous for the three gene products. The next 184 amino acids comprise the second region, which is characterized by divergence of the 4.5 kb-gene from the 5.3 kb- and 6.6 kb-genes. This pattern changes for the next 322 residues of region three, when the 5.3 kb and 4.5 kb-genes are virtually identical and the 6.6 kb-gene only shows a 63% homology with the other two genes. The fourth region includes the 367 amino acids which make up the C-terminal region. This region of the genes is highly conserved in all the genes sequenced to date.

The HD-2 gene

A fourth 130-kD protein gene was recently isolated from B. thuringiensis subsp. thuringiensis (Brizzard, B. and Whiteley, H., 1988). The open reading frame codes for 1228 amino acids. The overall similarity with the "4.5" kb-gene product at the amino acid level is 72.9% (11 gaps being necessary to maximize similarity). With similar parameters one obtains 71.7% similarity against the "5.3" kb-gene product (9 gaps) and 71/6% against the "6.6" kb-gene product (12 gaps). The amino terminal half of the protein is much less similar

to the HD-1 proteins than is the carboxy terminal. Compared to the "4.5" kb-gene product, for instance, one finds 56.5% similarity in the first half, versus 88.8% in the second half (Brousseau, R. and Masson, L., 1988). The distribution of the cysteines follows the trend of the three HD-1 genes in that only two cysteines out of 16 are located in the first half of the protein.

The mosquito factor (P2)

B. thuringiensis subsp. kurstaki HD-1 contains a mosquitocidal protein (called P2) in addition to the well studied 130 kDa protein discussed above (P1). Donovan, W., Dankocoki, C., Gilbert, M., Gawron-Burke, M., Croat, R. and Carlton, B., (1988) sequenced the gene for this 66 kDa protein. The gene codes for a protein of 590 amino acids which is very distantly related to other known B. thuringiensis crystal protein sequences. The similarities seen are in the N-terminal region which is necessary for toxicity. This may indicate an important conserved region.

B. thuringiensis subsp. entomocidus has been reported to have five genes, two of which belong to the "4.5" kb-gene type described previously (Visser, B., van der Salm, T., van der Brink, W. and Folkers, G., 1988). The gene sequence of one of the remaining genes is published (Hondée, G., van der Salm,

T. and Visser, B., 1988) and it shows "extensive homology" with the 4.5 kb gene type isolated from B. thuringiensis subsp. kurstaki HD-1 downstream of the proteolytic cleavage site (C-terminal). No homology is reported between this gene and the "5.3" kb- and "6.6" kb-gene types and the B. thuringiensis subsp. kurstaki HD-2 gene.

Protein Structure

The terms "crystalline inclusion" or "crystal protein" come from the bipyramidal shape which is usually observed when the protein is viewed through a phase contrast microscope (Figure 1). The individual subunits which make up the crystal are dumbell or rod-shaped with a molecular weight of several hundred thousand daltons (Norris, O., 1971).

The major components of the crystal are single chain polypeptides with molecular weights ranging from 130 kDa to 140 kDa (protoxin) held together in the crystal by disulfide bonds (Huber, H. and Lüthy, P., 1980). The amino acid composition of this protein does not show an unusual distribution of residues (Table I). The amount of carbohydrate present in the crystal has never been established, but has been reported as low as 0.4% and as high as 4% (Huber, H. and Lüthy, P., 1980; Fast, P., 1981).

FIGURE 1

B. thuringiensis subsp. kurstaki HD-1 crystal viewed under the phase contrast microscope.

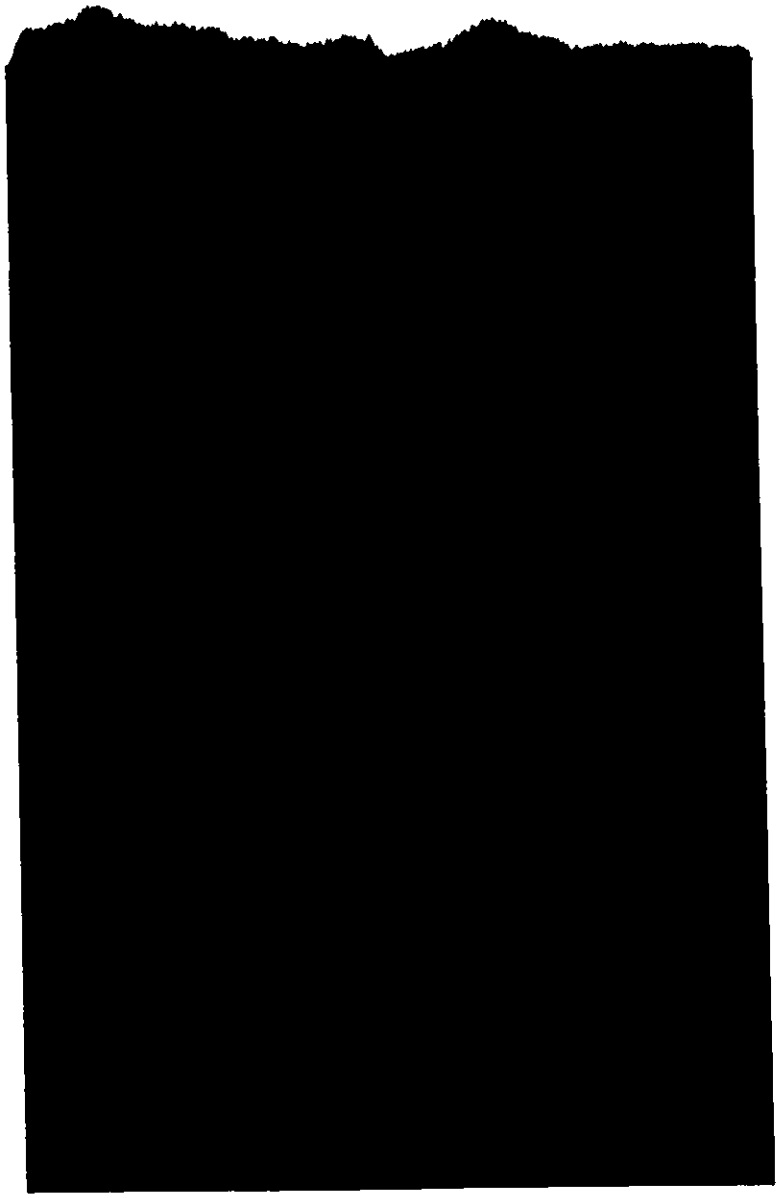


TABLE 1

The Amino Acid Composition of the B. thurigiensis subsp. kurstaki HD-1 Crystal Protein

Amino Acid	HD-1 Number	HD-1 (percent)
Ala	64	(5.44)
Val	81	(6.89)
Leu	102	(8.67)
Ile	71	(6.04)
Pro	56	(4.76)
Met	9	(0.77)
Phe	54	(4.59)
Trp	18	(1.53)
Gly	80	(6.80)
Ser	86	(7.31)
Thr	74	(6.29)
Cys*	17	(1.45)
Tyr	52	(4.42)
Asp	141	(11.99)
Glu	141	(11.99)
Lys	33	(2.81)
Arg	75	(6.38)
His	22	(1.87)

*Determined as cysteic acid (Hirs, 1956).
Calculated as moles/1176 moles; average of 5 determinations with estimated error of less than 7% for all amino acids.

Raman spectroscopic studies gave the first secondary structure analysis of the crystal protein from B. thuringiensis subsp. kustaki HD-1 and HD-73 (Carey, P. et al., 1986). It was found that the crystal protein is approximately 54% unordered, 25% α-helix and 21% β-structure. These results are in agreement with the secondary structure predictions based on the gene sequence carried out by Whitely, H. and Schnepf, H., et al. in 1986.

The protoxin can be divided into two parts of approximately equal molecular mass. The N-terminal portion is proteinase resistant and the C-terminal half is proteinase sensitive. The cleavage of the C-terminal portion is one of the events required for the activation of the protoxin. The fact that the C-terminal half contains almost all the cysteine residues and that its sequence is highly conserved suggests that it plays a significant role in establishing the structure of the crystal (Höfte, H. and Whiteley, H., 1989). Not only are the cysteines concentrated in this part of the protoxin, but also the majority of the lysine residues are found in this region. The function of this portion of the molecule is not clearly established. Andrews has suggested, on the basis of deletion experiments, that the C-terminal half is needed to give the N-terminal toxin its proteinase resistance (Andrews,

L., Bibilos, M. and Bulla, L. Jr., 1985).

The proteinase resistant fragment which makes up the toxin is derived from the N-terminal region of the protoxin by proteolytic cleavage at positions 29 and 623 (Bietlot, H., Carey, P., Choma, C., Kaplan, H., Lessard, T. and Pozsgay, M. 1989). The toxin from B. thuringiensis subp. kurstaki HD-73 was extensively characterized by Bietlot, H., et al. (1989). Raman spectroscopic analysis gave values of 20% α-helix, 35% β-sheet and 45% unordered structure for the secondary structure of the toxin. Based on the gene sequence, the toxin can be separated into "three subdomains: a relatively hydrophobic domain (resiudes 1 to 279), a variable domain (residues 280 to 460), and a toxic boundary domain (residues 461 to 614)" (Andrews, R., Jr., Faust, R., Wabiko, H., Raymond, K. and Bulla, L. Jr., 1987).

The mode of action of the B. thuringiensis toxin

The protoxin, when injested by susceptable larvae, becomes solubilized in the midgut due to the high pH of 8-12 (Jaquet, F., Hütter, R. and Lüthy, P., 1987). Proteinases act on the solubilized protoxin to produce the proteinase resistant core (toxin).

The highly specific nature of the action of the toxin towards insects is a strong indication of a specific recognition system. The toxin has been shown to act on the membrane of the midgut epithelium (Fast, P. and Donaghue, T., 1972 and Fast, P., Murphy, D. and Sohi, S. 1978) as well as on the surface of susceptible insect cells in culture (Knowles, B. and Ellar, D., 1987). The effect of the toxin is rapid. Within minutes, the larva's gut and mouth are paralysed leading to feeding inhibition. At the cellular level, the midgut epithelium columnar and goblet cells begin to swell and undergo granulation. Disintegration of the microvilli and cell lysis result in extensive damage to the midgut and eventually lead to insect death.

The mode of action of the B. thuringiensis toxin is an area of extensive research. To date two models which account for the action of the toxin have been proposed. Himeno, M., 1987; Himeno, M., Koyama, N., Funato, J. and Komano, T., 1987 found that the toxin-induced cell swelling was dependent on the Na^+ and K^+ concentrations in the external medium. The model they propose is that the toxin induces a cascade of biochemical events which ultimately leads to the following events: A) Na^+ influx through Na^+ channels, B) K^+ efflux through K^+-leak chanels, and C) Na^+/K^+ pump stimulation by

N^+/K^+-ATPase in the membrane. This causes a net influx of Na^+ and K^+ leading to the swelling and bursting of the cell. No conclusive proof has been provided to show that the actions of the toxin are responsible for an effect on K^+ permeability, on the Na^+/K^+ pump, or whether the action is due to ion channels in the membrane. The second model was suggested by Knowles, B. and Ellar, D. (1987). It involves a more general disruption of the permeability controls of the cells. These workers postulate that after binding of the toxin to a specific cell-surface receptor, small pores in the plasma membrane are created. Ions and small molecules then equilibrate across the membrane leading to cell lysis.

Proteinase activity in the B. thuringiensis crystal

The early literature suggests that the molecular mass of the polypeptides found in intact crystals varied considerably. When samples of protoxin were subjected to ultracentrifugation, a molecular mass of 177 kDa was obtained, while gel filtration chromatography gave a molecular mass in excess of 500 kDa for the same sample. The molecular mass reported from SDS-PAGE gel ranged from 157 kDa to 170 kDa. Ultracentrifugation in the presence of denaturing agents gave values ranging from 80 kDa to 10 kDa (Huber, H. and Lüthy, P., 1982). Many authors also report crystal consisting of

polypeptides ranging in molecular mass from 1,000 Da (Fast, P.G. and Martin, W. 1980; Saylers, V., Aronson, J. and Rosenthal, A., 1970) to 70 kDa in addition to the large molecular mass material mentioned above. Because the techniques used to prepare solubilized crystal proteins in these reports often involved harsh conditions such as extremely high pH and denaturing conditions, the data from these early experiments need re-examination.

Chestukhina, G., Zalumin, I., Kostina, L., Kotova, T., Katrukha, S. and Stephanov, V. (1980) proposed that some of the differences seen in molecular mass could be due to limited proteolysis. Various sorts of proteolytic activities are produced by the Bacillus family and several of the enzymes are synthesized in large amounts during the early stages of sporulation (Andrews, R.E. et al., 1985). The proteolytic processing of proteins during the sporulation cycle is well known. Modification of the RNA polymerase in B. thuringiensis subsp. thuringiensis was demonstrated by Ledadet, M. et al., 1977; Cheng Yih-Shyun and Aronson, R. (1977) showed that precursor proteins of the spore envelope of Bacillus cerus were modified by proteases before their incorporation.

Several reports suggest that the crystal associated proteases are of the serine, leucine, metalo or sulfhydyl classes (Bulla, L.A., Kramer, K. and Davidson, L. 1977; Chestukhina, G., Zalunin, I., Kostina, L., Kotova, T., Katrukha, S., Lyublinskaya, L. and Stepanov, V., 1978; Chestukhina, G., et al., 1980). A study of intracellular and extracellular protease activity during the life cycle of the bacteria (Chestukhina, G., Kotova, T., Zalunin, I. and Stepanov, V., 1979) showed that intracellular enzymes probably do not participate in the proteolytic processing of the crystal or of the spore proteins. Extracellular enzymes present between the inner- and outer-membranes of the cell as well as any enzyme specifically produced during sporulation are thus the likely candidates for the proteolytic degradation of the crystal protein.

Photoinstability of the crystal protein

Commercial preparations of B. thuringiensis sprayed in the field have a half life of 1.5 to 2 days (Ishigwo, T., Miyazone, M., 1982). Moreover purified B. thuringiensis crystals exhibit a marked loss in toxicity when exposed to direct sunlight for 4.5 hours (Morris, O.N., 1983). It has been shown (Morris, O.N., 1983) that U.V. light of wavelength 300 to 400 nm is responsible for the loss of toxicity and that

B. thuringiensis crystal preparations treated with compounds which absorb U.V. light have increased stability towards photoinactivation. The reported experiments used very rudimentary filters and had poor control over the amount of light used in the exposures. There is still at present no quantitative data on the effect of exposure to sunlight or precise information on the wavelength of light which causes the inactivation. However, it is evident from the available information that sunlight renders the protein non-toxic to the insect larvae.

The effect of light on protein remains an open question. It has been demonstrated that chromosomal DNA is the primary target in U.V. irradiation of bacterial cells. Certain types of primary lesions are amenable to in vivo repair procedures including the rejoining of single strand breaks, excision of thymine dimers and other base alterations, and dimer cleavage by photo reactivation. Recent studies on protein involved in DNA repair as well as on protein in tissue which is constantly bathed in radiation over a long period of time indicate that direct protein involvement may be more important than previously believed.

Photodynamic effects have been observed in virtually all classes of organisms ranging from inhibition of protozoan and bacterial growth to human photodynamic diseases such as erythropoetic protoporphyria. A large body of literature exists on the inactivation of many proteins, including 38 different enzymes (Grossweiner, L.I., 1976) as well as a number of functional proteins including hormones, antibodies, heme proteins, toxins and venoms.

Photosensitizing agents include compounds such as acridines, flavines, thiazine, xanthenes, and antraquinone. The compounds which are biologically important include xanthenes, acridines thiazines as well as some prophyrins. Virtually all photodynamic sensibilizing agents are fluorescent (Grosswiener, L.I., 1976). Many studies have shown that the triplet state of the outer electron of the chromophore is involved in the primary process or excitation and that the energy is transfered from the chromophore to oxygen to form singlet oxygen (O_2). Singlet oxygen is a very powerful oxidizing agent whose efficiency at destroying various amino acid side chains is limited by physical quenching of the dye triplet state and energy transfer to the oxygen molecule (Grossweiner, L.I., 1986).

There are two general mechanisms by which damage from photochemical irradiation can occur. The direct method of destruction involves the direct absorption of a photon by the chromophoric group on the side chain of the amino acid. This type of destruction depends on the wavelengths of the incident light. The other broad phenomenon is "photodynamic action", where the system is irradiated with light which is absorbed by an external or conjugate moiety at wavelengths which are not absorbed by the side chain of the amino acid. Both cases can lead to extensive physical and chemical alterations in protein absorption and fluorescence spectra, optical rotation, electrophoretic pattern, solubility, sedimentation, light scattering, viscosity, heat sensitivity, digestibility, and residue destruction (Grossweiner, L.I., 1976). The biological damage which results may be observed in alterations of antigenicity and antibody reactivity as well as in changed biological activity.

Light is absorbed selectively by the different chromophores, and hence the effects are strongly dependent on the incident wavelength, the chemical structures of the target molecule and the microenvironment of the chromophore and the protein in general. In the case of large proteins it is usually found that the permanently damaged residues are the

amino acid chromophores, histidine, tyrosine and tryptophan. Not all residues of a certain type are damaged in a given protein because of the highly specific effect of the microenvironment and the tertiary structure. This fact makes it very difficult to compare results obtained from flashphotolysis experiments on aqueous solutions of each amino acid or peptides with experiments carried out on intact proteins.

Pure protein containing no cofactor or prosthetic groups has no major electronic transition above 290 nm and sunlight at the earth's surface does not contain light with wavelengths below 300 nm. The direct destruction of tryptophan, histidine or tyrosine side chains in the B. thuringiensis crystal by direct absorption of a photon is thus a very unlikely event (Pozsgay, M., Fast, P., Kaplan, H. and Carey, P., 1987). Since the direct destruction of the imidazol, indole or phenol side chains appears unlikely, the destruction of these side chains probably occurs through a photosensitizing agent (Pozsgay, M., et al. 1987). As previously described, this mechanism probably involves the absorption of light by an endogenous chromophore (probably non-covalently bound to the crystal) and the transfer of energy to a neighboring O_2 molecule creating singlet oxygen.

The chromophore responsible for the absorption of light in B. thuringiensis has not been identified or characterized. Its existence was first reported by Bulla, L.A., et al. 1977 who found that in a neutralized alkaline-solubilized crystal preparation, an unusual ultraviolet-absorbing chromophore was detected. The absorption spectrum revealed the presence of tryptophan and tyrosine as expected, in addition to a novel ultraviolet-absorbing chromophore which is dialyzable. Harms, R., Martinez, D. and Griego, V. (1986) reported that crude B. thuringiensis spore and crystal preparations had a large absorption peak in the region of 408 nm to 420 nm. These peaks are indicative of porphyrins, which are known to be efficient photosensitizers.

Sulfhydryls

The crystal protein from every subspecies of B. thuringiensis requires high pH's or thiol reagents for solubilization (Nickerson, K. 1980; Huber, H., Lüthy, P., Ebersol, D.H. and Cordier, J-L. 1981; Couche, G., Pfannenstiel, M. and Nickerson, K. 1987). The need for conditions under which disulfide bridges are ruptured is a good indication that the individual protoxin molecules are held in their crystalline form by a network of disulfide bridges. These are responsible for the extreme insolubility

of the crystal which is comparable to the solubility of keratin (cysteine content 10%). The crystals cysteine content is 1.3% which is similar to that of BSA (Huber, H. and Lüthy, P., 1981). There is at present no report in the literature on the arrangement of the cystine residues in any crystal from any subspecies of B. thuringiensis. Furthermore, no direct analysis for cystine or cysteine is published for any kurstaki strain.

Nickerson, K. (1980) has provided evidence that the disulfide bridges are important in determining the structure and properties of the B. thuringiensis crystal. The fact that disulfide bonds are present does not prove that they are interchain and that they are responsible for the extreme insolubility seen, however, several pieces of evidence suggest that they are. The refractile properties of the crystals, as seen under the phase contrast microscope, are lost when the crystals are placed in 8M urea and allowed to swell. They then reappear when the urea is removed, and during this procedure no protein is released into solution (Nickerson, K. 1980). During the early stages of synthesis, crystal protein can be extracted at mild alkaline conditions in the absence of thiol reagents. After a period of time which corresponds to the onset of refractibility, thiol reagents and higher pH's are

needed to solubilize the crystal (Nickerson, K., 1980). The mild alkaline conditions used are enough to break interchain disulfide bonds since they are thermodynamically weaker than their intrachain counterparts (Nickerson, K., 1980) and this would seem to indicate that interchain disulfides are formed first, followed by the formation of any intrachain bonds.

This hypothesis is supported by immunological evidence which shows that one of the primary antigens of the crystal protein appears as soon as the crystal appears. A second antigen appears only with the onset of refractibility suggesting a "maturing" process (Nickerson, K., 1980).

Aim and Rationale of the present study

B. thuringiensis is becoming the major insecticidal element in commercial sprays used to fight forestry and agricultural pests within this country. From the preceding review of the literature, it is evident that precise information on the structure of the main insecticidal element, the crystal protein, is not available. Accordingly, the present work was undertaken to study various aspects of the structure of the crystal protein. The objectives of the work presented in this thesis are three-fold; (1) to study the proteolytic activity associated with the crystal, (2) to quantify the destruction of amino acid side chains upon solar

irradiation and (3) to quantify and characterize the various forms of the cysteine residues as well as to determine the alignment of the disulfide bridges.

The proteolytic activity associated with the crystal was studied by monitoring the degradation of the crystal protein by gel permeation chromatography. Also, a novel method for N-terminal analysis was developed to study the proteolytic activity during the solubilization of the crystal.

The objective of the series of experiments on photostability was the quantification of the destruction of various amino acid side-chains in three different crystal types. Amino acid analysis was used to quantify the destruction of tryptophan, histidine, tyrosine and methionine and this destruction was correlated to the loss of toxicity.

The routes used to obtain the objectives of the third study were the use of amino acid analysis to quantify the cystine, cysteine and free sulfhydryl groups, competitive labelling to characterize the chemical reactivities of the cysteine, and a modified diagonal electrophoresis procedure was used to determine the alignment of the disulfide bridges.

MATERIAL AND METHODS

Growth and Purification of Protein Crystals

Bacillus thuringiensis subsp. kurstaki HD-1 and Bacillus thuringiensis subsp. entomocidus were grown and purified by the method described by Fast et al., 1972. The crystals were further purified by the method developed by Carey et al., 1986 and stored at 4°C in water at pH 6.0. Crystals were shown to be free of contaminating proteases by a lack of proteolytic activity toward denatured [^{14}C]methylated hemoglobin (Rice, R. and Means, G., 1971).

Solubilization of the Protein Crystal

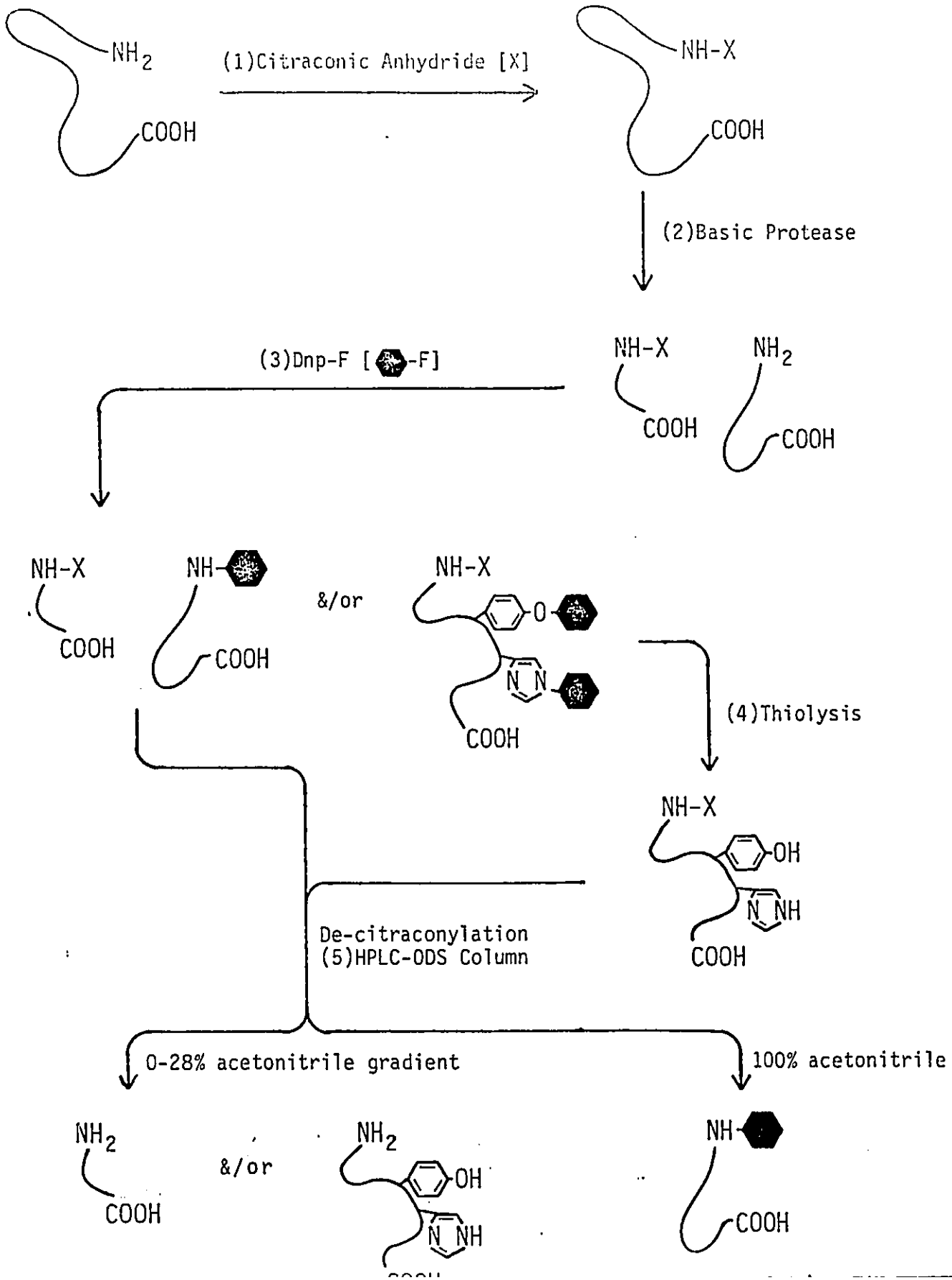
A sample (500 µl) of protein crystal suspension (10-15 mg of protein) was resuspended in 2 ml of a solution containing 2% 2-mercaptoethanol at pH 10.5. The pH was maintained at 10.5 while the solution was magnetically stirred for 1 h. Any insoluble material was then removed by centrifugation.

Solubilization of the Protein Crystals in the Presence of Urea

Crystals were resuspended in an 8M urea solution at pH 3. The solution was stirred for 1 h before the pH was raised to 10.5 and 2-mercaptoethanol added in a final concentration of 2%. The rest of the solubilization procedure is as

FIGURE 2

Reaction scheme for the isolation of N-terminal peptides.


NH2
COOH
(1)Citraconic Anhydride [X]
NH-X
COOH
(2)Basic Protease
NH-X
COOH
NH2
COOH
(3)Dnp-F [-F]
NH-X
COOH
NH
COOH
&/or
NH-X
O
N N
COOH
(4)Thiolysis
NH-X
OH
N NH
COOH
De-citraconylation
(5)HPLC-ODS Column
0-28% acetonitrile gradient
100% acetonitrile
NH2
COOH
&/or
NH2
OH
N NH
NH
COOH

described above.

Purification of the Crystal Protein by Column Chromatography

Soluble protoxin was placed on a Sephacryl S-300 (Pharmacia) gel permeation column of dimensions 120 cm x 1.6 cm. The sample was eluted with a running buffer consisting of 0.1% 2-mercaptoethanol, 50 mM Tris and 1mM EDTA at a flow rate of 1 ml per minute. Fractions (3 ml) were collected and the absorbance of these fractions was monitored at 280 nm.

Column Calibration

Calibration of the column was carried out using a Pharmacia MW-GF-200 molecular mass standard kit in accordance with the manufacturer's instructions.

Detection of the N-terminal Peptides

The scheme used to detect the N-terminal peptides was developed by Kaplan, H. et al. (1986) and is illustrated in Figure 2. The method was used on protoxin solutions which were solubilized in the presence and absence of urea. Citraconylation was carried out in 8M cyanate-free urea at a pH of 9.0 by the addition of six aliquots of 10 µl of citraconic anhydride. After dialysis, the crystals were digested with either elastase, thermolysin or pronase (100:1 w/w) in 1 ml 1% ammonium bicarbonate at 37°C for 24 h. The reaction was stopped by lyophilization. Dinitrophenylation

(100 µl of DNP-F 50% in acetonitrile for 18 h at 22°C), was carried out in 1 ml of 1% N-methylmorpholine. This solution was then acidified and extracted with 3 x 5 ml of ether. Thiolysis was carried out by using the procedure of Shaltiel, S. (1967). The peptide mixture was extracted four times with 5 ml ethyl acetate, dried, and made up in a minimum volume of 0.01N HCl. The peptides were then applied to an altex ultrasphere - ODS (0.45 x 25 cm) column running at 1 ml/min in 0.01N HCl. After 5 min of 0.01N HCl, the percentage of acetonitrile was raised to 38% (v/v) over a period of 30 minutes after which the acetonitrile was increased to 100% (v/v) over 3 min. The fractions were monitored at 210 nm.

Irradiation Study

All solar irradiations were carried out under an Oriel (Stamford, C.T.) solar simulator with a light filter combination chosen to deliver a solar spectrum equivalent to air mass 1.5 (globol). The total light falling on the sample area was measured as 70 mW cm^{-2}. The temperature at the sample was 27°C with room temperature set at 22°C (Carey, P. et al., 1986).

Crystal suspension (1 mg) was spread on a microscope cover slip (1.2 cm x 1.2 cm) and allowed to dry. After exposure to light under the solar simulator, the glass cover

slip was cut in half and placed into pyrex tubes. The hydrolysis was carried out by either of the following methods.

Amino Acid Analysis

Samples were hydrolyzed in 1 ml of 6N HCl in the presence of phenol and norleucine. The tubes were sealed under vacuum and hydrolyzed at 110°C for 24 h. After lyophilization, the samples were resuspended in a pH 2.1 sodium acetate buffer and analyzed on a Technicon automatic amino acid analyser using a ninhydrin detection system.

Tryptophan analysis was carried out by hydrolysing the protein in 500 µl of 4N methane sulfonic acid in the presence of 0.2% 3-(2-aminoethyl)indole. After 24 h of hydrolysis at 110°C *in vacuo*, the sample was placed on the amino acid analyser using an extended pH 7.5 sodium citrate buffer.

Bioassays

All bioassays were performed by the Forest Pest Management Institute in Sault Ste Marie. Toxicity of irradiated *B. thuringiensis* subsp. *kurstaki* HD-1 crystals was determined by force feeding fifth instar larvae of silkworm, *bombyx mori*, and assessing mortality after 24 h. The LD_{50} was calculated by the probit analysis method of Finney (1970).

Determination of Cysteine Content

The cysteine content of the crystal was determined by two methods: 1) a sample (1 mg) of crystal was performic acid oxidized (Hirs, C., 1956) and cysteic acid was quantified by amino acid analysis, 2) the same amount of crystal was solubilized with 10% (v/v) 2-mercaptoethanol, 8M urea, 0.2M tris pH 8.6. Iodoacetic acid (26.8 mgs in 100 µl of 1N NaOH) was added and the reaction allowed to proceed for 30 min. After dialysis and hydrolysis, carboxymethylcysteine (CM-Cys) was quantified by amino acid analysis.

Determination of Cystine Content

Purified crystals (1 mg) were hydrolysed in 1 ml of 6N HCl in the presence of phenol and norleucine, and cystine was quantified by amino acid analysis.

Quantification of the Free Sulfhydryls Present in the Crystal Protein

The number of free sulfhydryls was quantified by reacting any free sulfhydryl groups with iodoacetic acid as described in Bietlot, H. et al., (In press).

Identification of Free Sulfhydryl Peptides

To identify free sulfhydryl-containing peptides, crystal protein was reacted with ^{14}C iodoacetic acid, digested and run

on pH 6.5 electrophoresis as described in Bietlot et al., (In press).

Determination of Disulfide Bridge Alignment

The procedure used to determine the disulfide bridge alignment was that of Brown and Hartley (1966) which was modified to obtain sufficient quantities of peptide for detection of off-diagonal peptides. B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus crystals (50 mg) were suspended in 10% formic acid and digested with 2% pepsin (w/w) at 37°C for 24 h. The solution was then diluted to a concentration of 5% formic acid and lyophilized. The peptide mixture was dissolved in a minimum volume of pH 6.5 buffer and applied across a 10 cm band on Whatman 3MM chromatographic paper along with markers of dansyl arginine and dansyl chloride. Electrophoresis was carried out at pH 6.5 for 40 min at a voltage gradient of 60V/cm.

A modification to Brown and Hartley's original procedure was needed in order to obtain sufficient off-diagonal peptides for detection. Instead of a 3cm strip being subjected to performic acid oxidation, the material along our 10 cm band was concentrated prior to oxidation. This was achieved by stitching pieces of Whatman 3MM paper along either side of our 10 cm band and allowing buffer to flow into the paper from

either side meeting in the middle of the 10 cm band and concentrating the peptide. After drying, a 3 cm band was cut out and subjected to oxidation. Oxidation was carried out in a partially evacuated dessicator containing a freshly prepared mixture of 35 ml of 98% (v/v) formic acid and 10 ml of 30% (v/v) hydrogen peroxide. Ten ml of formic acid and five ml of hydrogen peroxide were added together after 1 h and 2 h of the four hour incubation. The oxidized guide strip was removed from the dessicator and the fumes allowed to evaporate.

The strip was then stitched across the center of a Whatman 3MM sheet at a 90° angle to the original electrophoresis and subjected to electrophoresis at pH 6.5 for 40 min with a voltage gradient of 60V/cm. The electrophoretogram was then stained with cadmium ninhydrin to give a diagonal "fingerprint". In order to clearly assign disulfide bridges arising from peptides in the neutral band (N), these peptides must be separated prior to performic acid oxidation. This was achieved by running the neutral band at pH 2.1 at a voltage gradient of 60V/cm for 40 min. After concentrating the peptides as described above, performic oxidation was carried out followed by electrophoresis at pH 6.5 at a voltage gradient of 60V/cm for 40 min. The neutral

band "fingerprints" were visualized by staining the electrophoretogram with cadmium ninhydrin reagent.

Peptide Isolation

The peptides were isolated according to the procedure of Brown and Hartley (1966). After elution from the paper, peptides were further purified by reverse phase HPLC. The gradient used ranged from 0-50% acetonitrile/0.01N HCl over 60 min.

Reaction of Solubilized Protoxin with Iodoacetic Acid and Iodoacetamide

The solubilized protoxin (1mg) in 100 µl of 0.2M sodium metaborate, pH 9.0 was reacted with iodoacetic acid (26.8 mg in 100 µl of 1M NaOH) or iodoacetamine (27 mg) for 5 min. CM-Cys was quantified by amino acid analysis after dialysis and acid hydrolysis.

Solubilization of Crystals with Mercaptoethanol

Crystals (500 µg) were suspended in 0.1M CAPS buffer pH 10.5, containing amounts of 2-mercaptoethanol varying from 0 to 2% v/v and incubated for five minutes at 25°C. An aliquot (5 µl) of iodoacetate (10 mg/ml, pH 10.5) was added and the sample run on non-reducing PAGE electrophoresis.

Gel Electrophoresis

Gels were run on a Pharmacia Phast electrophoresis system with preformed gels and other materials supplied by Pharmacia. Samples were dissolved in sample buffer, placed in a boiling water bath, cooled and then applied to 10-15% gradient gels.

Preparation of [^{14}C]DNP-Derivatives

S-[^{14}C]-DNP-cysteine and [^{14}C]-DNP-alanylalanine were prepared by reacting the Nα-acetyl derivatives of these amino acids with [^{14}C]Dnp-F as described previously (Hefford, M-A. et al., 1985). The purification procedure was the same as that described by Hefford, M-A. et al. (1985) except that the final purification step was C18 reverse phase HPLC. The [^{14}C]DNP-alanylalanine was eluted using the following solvents: 0.01N HCl-17% acetonitrile for 6 minutes; 0.01N HCl-25% acetonitrile for 4 minutes followed by 0.01N HCl-60% acetonitrile for 30 minutes. The S-[^{14}C]DNP-cysteine was eluted isocratically with 0.01N HCl-60% acetonitrile.

Competitive Labelling of Protoxin

Aliquots (1.0 ml) of a stock solution containing 0.113 mg/ml (3.57×10^{-7}M) of protoxin and alanylalanine (7.50×10^{-5}M) in 5mM N-methylmorpholine-5mM sodium borate were equilibrated at 25°C in a thermostatted water bath. The pH was

adjusted to the desired value between 7 and 10 using either 1N NaOH or 1N HCl. An aliquot (50 µl) of acetonitrile containing [^{3}H]Dnp-F (20.8 nmol, Sp. activity 16.6 Ci/mmol) was added with vigorous stirring and the reaction allowed to proceed 18 h in the dark. Concentrated HCl was added to bring the pH to 2.0.

The following was added to each sample: An aliquot (1.0 ml) of a 25% acetone solution containing 2500 dpm of [^{14}C]DNP-alanylalanine and S-[^{14}C]DNP-cysteine and 0.03 mg of each unlabelled DNP-derivative as carrier. The acetone was removed by evaporation and the samples were hydrolysed in 6M HCl for 18 h at 110°C in vacuo.

Purification of [^{3}H][^{14}C]DNP-Derivatives

The [^{3}H]/[^{14}C]DNP-derivatives were isolated and purified as described previously (Hefford, M-A. et al., 1985), with the only change being that the final purification step was carried out by C18 reverse phase HPLC.

Liquid Scintillation Counting

Samples were dissolved in 0.1 ml of 0.01N HCl and added to 10 ml of aquasol-2. Scintillation counting was carried out on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter.

Analysis of Titration Curve

A non-linear least square regression (Enzferher, Bioroft, Milton, N.J.) was used to fit the reactivity data to a titration curve.

RESULTS

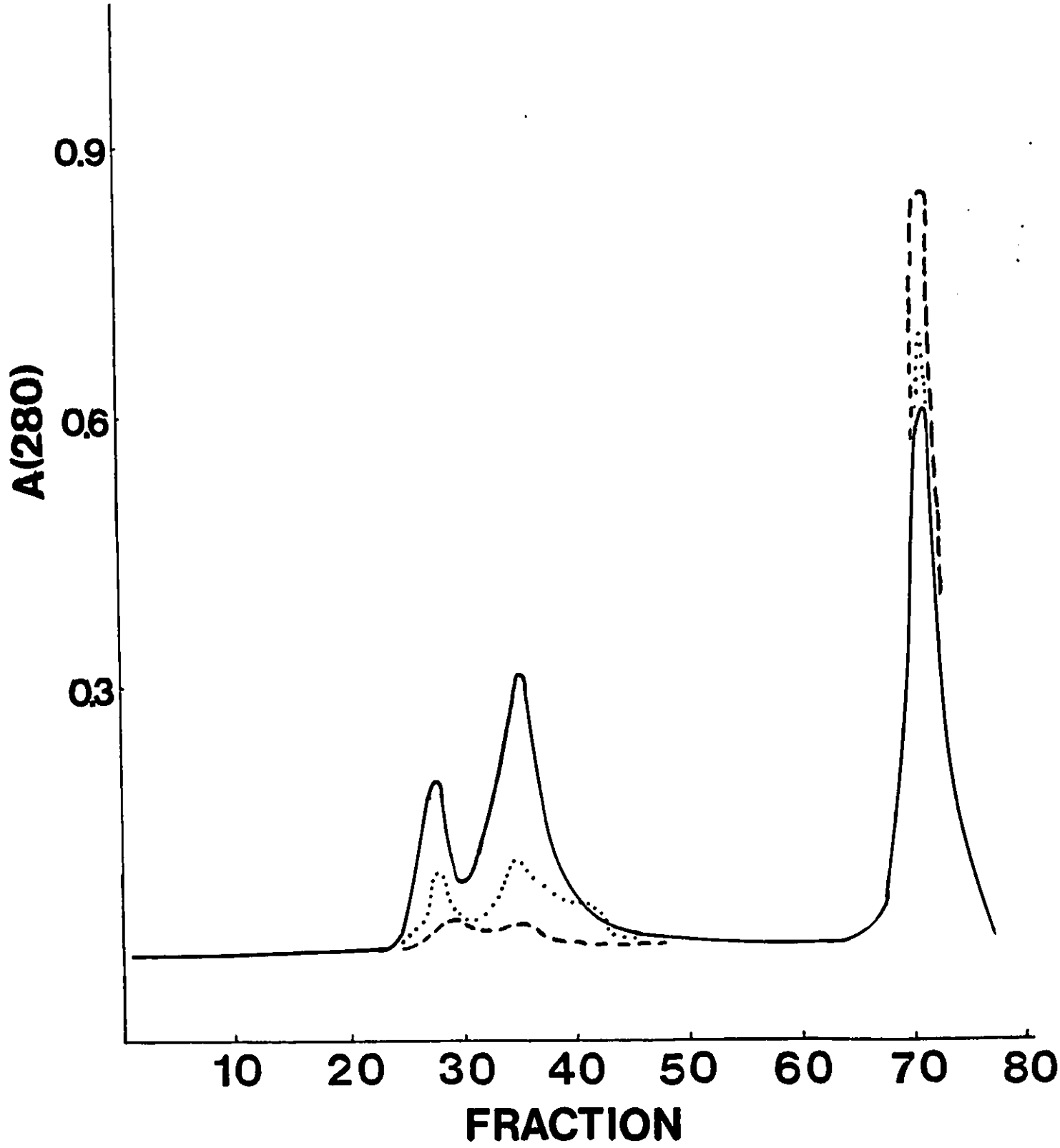
Proteolysis of Crystal Protein

The first series of experiments was carried out using B. thuringiensis subsp. kurstaki HD-1 crystals which were purified by the methods of Fast, P. (1972). The crystal to spore ratio, as viewed under the phase contrast microscope, was approximately 60-80:1. (The method developed by Carey, P. et al., (1986) was used in subsequent experiments and it has a better than 1000:1 crystals to spore ratio). The aim of these experiments was to study the degradation of the crystal protein during storage and during its solubilization. Column chromatography was used to study the degradation pattern of the protoxin and to monitor the proteinase stability of the protein crystal preparation. N-terminal analysis using HPLC was used to study the proteolytic degradation of the protein crystal during the solubilization process customarily used.

The elution profile obtained from chromatography on a Sephacryl S-300 gel permeation column is shown in figure 3. This experiment was carried out three times over a period of 34 days: on the day after purification, twenty one days later, and finally after 34 days of storage in distilled water at 4°C. In each case there are three peaks in the profiles at fractions 27, 35 and 75 respectively. The first peak (fraction

FIGURE 3

Elution profile of solubilized crystal protein from B. thuringiensis subsp. kurstaki HD-1 on Sephacryl S-300. The experiments were carried out after 1 day of storage (solide line), after twenty one days (dotted time) and after thirty four days (dashed line).


0.9
0.6
0.3
A(280)
10
20
30
40
50
60
70
80
FRACTION

27) has an estimated molecular mass of 250 kDa when its elution volume is compared to that of known proteins. When the material is run on SDS Page, a band corresponding to the protoxin (molecular mass 130-kDa) is seen. If the crystal protein is applied to the same column in the presence of 8M urea and eluted with running buffer containing 8M urea, this peak disappears while the absorbance of peak two increases. The second peak (fraction 35) migrates with a molecular mass of 130, kDa on the column. The same molecular mass is obtained on SDS page. The third peak (fraction 75) coincides with the elution volume of the column and contains material of molecular mass less than to 10-kDa. When the fractions which contain peak three are pooled and run on a Sephadex G-50 column, (molecular mass range 1.5 - 30 kDa) the material elutes as a broad peak at the void volume. This peak contains the high concentration of 2-mercaptoethanol that was used in the initial solubilization of the crystal protein as well as degradation products. It was found that the absorbance at 280 nm of 2-mercaptoethanol increases with time and as such, the absorbance measured in this peak is not solely due to the absorption of "protein material".

The trend that is seen in each of these trials is a gradual reduction of the peaks containing high molecular mass material, and an increase in the amount of low molecular mass

material. After 34 days of storage, the amount of high molecular mass material is reduced by 80-90%. At no time were we able to see the accumulation of any material in the molecular mass range of 100 to 10 kDa. The fact that no material corresponding to intermediates of the degradation process is seen indicates that after initial cleavage, the products are rapidly broken down into very small peptides.

Noticeably missing from these elution profiles is the mosquito factor (molecular mass 66-kDa). It was expected that this material would appear at the same position as BSA but except for a negligeable amount which appears to be present in one run, no peak corresponding to this material is seen.

Proteolysis of the crystal protein was also demonstrated using reverse phase HPLC. The method developed by Kaplan, H. et al., (1986) is based on the rationale that after citraconylation of the free amino groups of the protein, followed by enzymatic digestion and reaction with fluorodinitrobenzene (Dnp-F), only peptides derived from the N-terminus will not contain a Dnp-group. After removal of the citraconyl group only N-terminal peptides will have a free amino group. The strong adsorption of Dnp-group retards the elution of all peptides on reverse phase HPLC except for the N-terminal peptide which does not contain a Dnp-group.

Figures 4A and 4B represent the HPLC profile as measured at 210 nm obtained for B. thuringiensis subsp. kurstaki HD-1. Figure 4A represents the profile of elastase digested crystals where proteolysis is not inhibited during the solubilization procedure. There are clearly many amino termini present in this case. This does not establish whether these multiple N-termini were obtained by proteolysis or if they represent many different polypeptides within the crystals. Figure 4B is the profile obtained when proteolysis is eliminated during solubilization. In this case proteolysis was eliminated with 8M urea at pH 3.0. There is only one amino terminus present, indicating that the crystal inclusion body is made up of a single polypeptide species which is rapidly degraded during the solubilization.

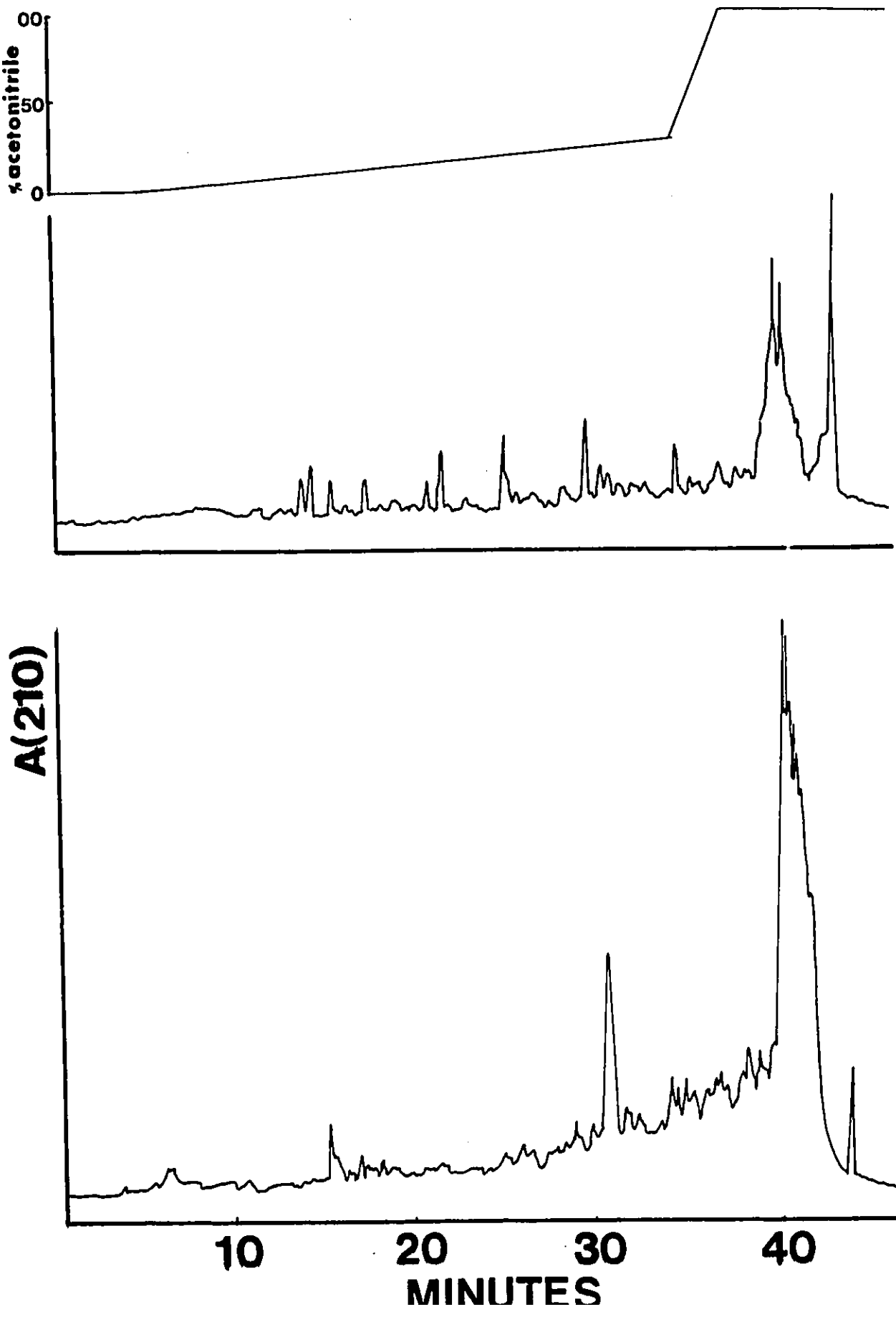
Irradiation Study

Amino acid analysis was used to quantify the destruction of tryptophan, histidine, tyrosine and methionine side chains. The data were obtained by calculating the ratios of the area of the residue in question to that of four amino acids stable to oxidation: leucine, phenylalanine, lysine and arginine. The ratios obtained from an irradiated sample were then compared to control samples and the residues remaining are expressed as a per cent of the control. Each data point is the average of two hydrolyses.

FIGURE 4

A) HPLC elution profile of B. thuringiensis subsp. kurstaki HD-1 crystal protein solubilized at pH 10.5 with 2% 2-mercaptoethanol and digested with elastase.

B) HPLC elution profile of B. thuringiensis subsp. kurstaki HD-1 crystal protein treated with urea at pH 3.0 prior to solubilization.


00
50
0
% acetonitrile
A(210)
10
20
30
40
MINUTES

Tryptophan analysis was carried out in 4N methane sulfonic acid as described in materials and methods. The results are tabulated in Table 2. In all three crystal preparations, there is a maximum decrease ranging from 40 to 45% in the tryptophan content. This loss corresponds to the destruction of seven or eight residues out of a possible 18. The rate at which this decrease occurs in each crystal type is slightly different. The decrease occurs at a faster rate in B. thuringiensis subsp. kurstaki HD-1 than B. thuringiensis subsp. entomocidus and Thuricide where the rates are virtually identical. In B. thuringiensis subsp. kurstaki HD-1, 90% of the loss of tryptophan residues is achieved by approximately 28 h of irradiation. This amount of destruction is not seen until 34 h of exposure to sunlight in B. thuringiensis subsp. entomocidus and in Thuricide.

The results obtained for histidine, tyrosine and methionine arise from samples hydrolysed in hydrochloric acid. The destruction of the imidazole side chain of the histidine residues is given in Table 3. There is a destruction ranging from 22% for the B. thuringiensis subsp. entomocidus strain to 18% for Thuricide with B. thuringiensis, subsp. kurstaki HD-1 undergoing a 20% decrease after 60 h of irradiation. This represents a destruction of five residues out of a possible 22 for B.

thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus.

Though there is very little difference in the total amount of histidine destroyed, the rate at which this destruction occurs appears to be different in each crystal type. B. thuringiensis subsp. entomocidus crystals undergo 90% of the total histidine destruction after 32 h of irradiation while B. thuringiensis subsp. kurstaki HD-1 and Thuricide crystals require 48 h of irradiation to reach the same level of destruction.

The destruction of tyrosine residues was also quantified and the results obtained are presented in Table 5. The trend observed in all three subspecies is very similar. There is a five per cent destruction of the total tyrosine content of the protein which represents the loss of two or three tyrosine residues out of the total 55.

The sulfur atom is also susceptible to oxidation by air or more potent oxidants such as peroxides and singlet oxygen. In the case of methionine, oxidation gives rise to methionine sulfoxide and then to methionine sulfone. The first step of this reaction is reversible under acid hydrolysis conditions while the reaction of methionine sulfoxide to methionine sulfone is not. Table 4 represents the changes in the methionine content due to solar irradiation. The method used

does not distinguish between methionine residues which were not oxidized and those which were re-generated from the sulfoxide form.

B. thuringiensis subsp. entomocidus shows the greatest loss of methionine at 14% while B. thuringiensis subsp. kurstaki HD-1 and Thuricide losses are 11% and 9% respectively. This represents a loss of approximately one residue in the protein from each of the three subspecies.

In order to establish the significance of the destruction of amino acid side chains, toxicity assays were performed in an effort to correlate destruction with loss of toxicity. The only available toxicity assays available were for B. thuringiensis subsp. kurstaki HD-1 crystal. The assays were performed by the Forest Pest Management Institute in Sault-Ste Marie on fifth instar larvae of silk worm, Bombyx mori, using the force feeding technique. Control samples of non-irradiated crystals were assigned a potency of one, and irradiated samples were expressed as fraction of the control.

As can be seen in Fig. 5, the loss of biological activity is complete after 8h of exposure to sunlight. A fifty percent loss in toxicity would correspond to the loss of less than one tryptophan. Therefore, it appears unlikely that the destruction of tryptophan can account for the solar instability of the crystal protein. Since the destruction of

TABLE 2

The destruction of tryptophan side chains by solar irradiation in B. thuringiensis subsp. kurstaki HD-1, B. thuringiensis subsp. entomocidus and thuriside

Time h	Subspecies		
	B. thuringiensis subsp. kurstaki HD-1	B. thuringiensis subsp. entomocidus	Thuricide
0	100	100	100
4	80	93	90
8	74	85	80
12	68	86	87
16	75	70	77
20	66	-	58
24	60	74	68.5
28	-	72	73
32	65	-	66
36	58	-	60
40	57	-	67
42	-	59	-
45	-	52	-
50	55	-	61
60	56	-	60
62	-	59	-

Calculated as a percentage of the non irradiated sample; average of 2 analysis with an estimated error less than 10%.

TABLE 3

The destruction of histidine side chains by solar irradiation in *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *entomocidus* and thuriside

Time h	Subspecies		
	B. thuringiensis subsp. *kurstaki* HD-1	*B. thuringiensis* subsp. *entomocidus*	Thuricide
0	100	100	100
4	98	98	100
8	93	93	96
12	88	86	92
16	83	-	95
20	89	-	93
24	92	84	93
28	85	-	89
29	-	78	-
32	86	80	85
36	86	-	84
40	83	-	85
42	-	80	-
45	-	74	-
50	79	79	81
60	-	-	84

Calculated as a percentage of the non irradiation sample; average of 2 analysis with an estimated error less than 10%.

TABLE 4

The destruction of methionine side chains by solar irradiation in *B. thuringiensis* subsp. *kurstaki* HD-1, *B. thuringiensis* subsp. *entomocidus* and thuriside

Time h	Subspecies		
	B. thuringiensis subsp. *kurstaki* HD-1	*B. thuringiensis* subsp. *entomocidus*	Thuricide
0	100	100	100
4	103	102	105
8	90	97	92
12	85	90	93
16	85	-	95
20	-	-	96
24	94	92	84
28	95	85	79
29	-	87	-
32	99	84	92
36	94	-	-
40	95	91	91
42	-	80	-
45	-	93	-
50	79	86	96
60	89	92	-

Calculated as a percentage of the non irradiation sample; average of 2 analysis with an estimated error of less than 10%.

TABLE 5

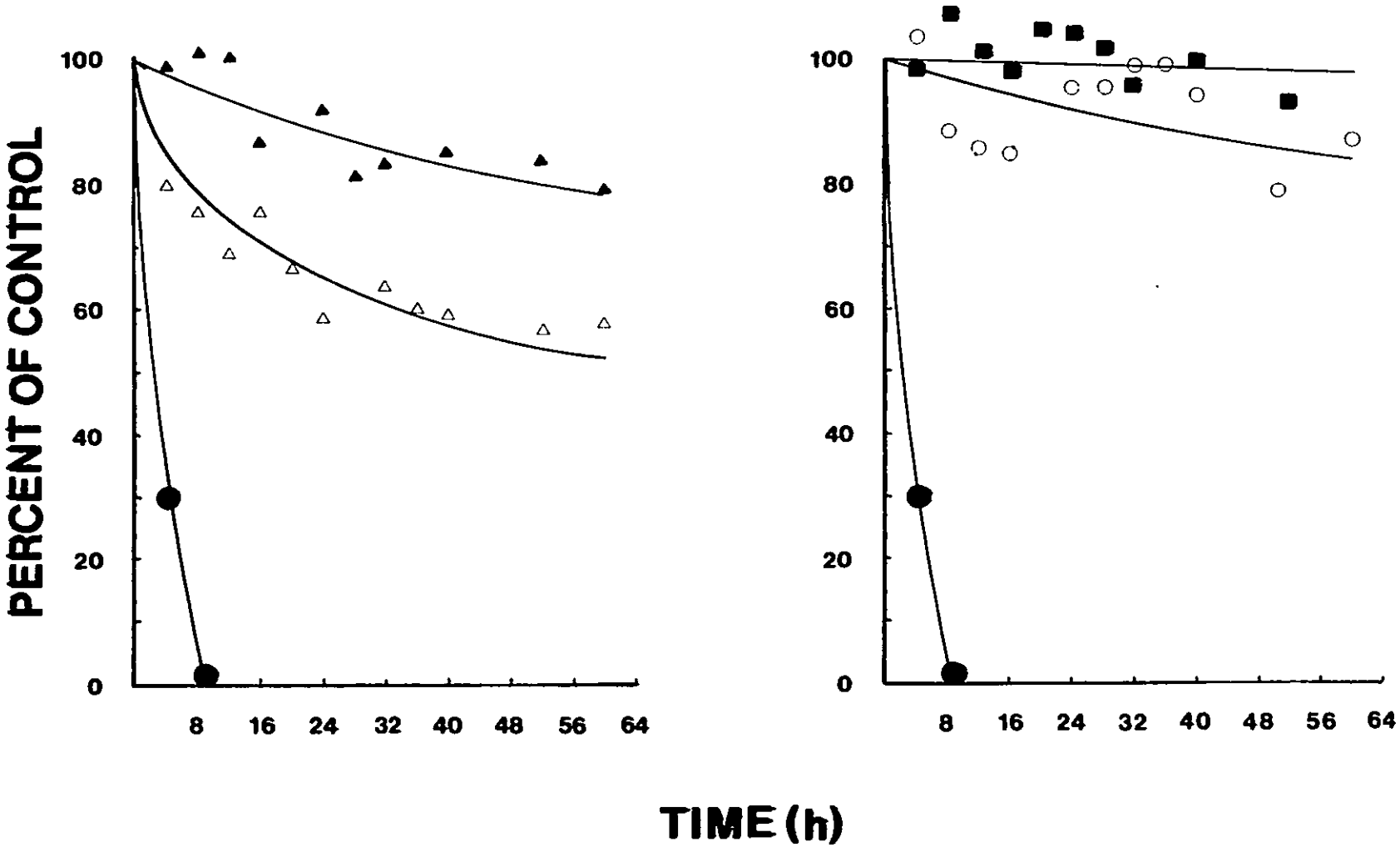
The destruction of tyrosine side chain by solar irradiation in B. thuringiensis subsp. kurstaki HD-1, B. thuringiensis subsp. entomocidus and thuriside

Time h	Subspecies		
	B. thuringiensis subsp. kurstaki HD-1	B. thuringiensis subsp. entomocidus	Thuricide
0	100	100	100
4	92	96	100
8	94	91	94
12	98	101	93
16	91	-	101
20	90	-	103
24	99	100	99
28	103	-	96
29	-	91	-
32	102	90	96
36	94	-	92
40	101	-	103
42	-	96	-
45	-	94	-
50	100	98	100
60	-	-	94

Calculated as a percentage of the non irradiated sample; average of 2 analysis with an estimated error of less than 10%.

FIGURE 5

The destruction of various amino acid side-chains by solar irradiation and the resultant loss in toxicities. The open triangles represents the destruction of tryptophan, the closed triangle the loss of histidine, the open circle, the loss of methionine the squares the destruction of tyrosine and the closed circles the less of biological activity.


PERCENT OF CONTROL
100
80
60
40
20
0
8
16
24
32
40
48
56
64
TIME (h)

tryptophan occurs at the fastest rate, it would then also seem highly unlikely that the destruction of histidine, tyrosine or methionine can account for the loss in biological activity.

Cysteine Content and Free Sulfhydryl Groups

The total number of sulfhydryl groups present in the crystal proteins from B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus was determined by the following three procedures: (i) hydrolysis of crystal in 6N HCl and quantification of cystine; (ii) performic acid oxidation and quantification of cysteic ácid after acid hydrolysis; (iii) reduction with β-mercaptoethanol, reaction with iodoacetate and quantification of CM-Cys after acid hydrolysis. The results are summarized in Table 6. Within the limits of the experimental error, we found no difference for the cysteine content of both subspecies. The cystine content confirms the deductions made in earlier reports that most, if not all, of the cysteine residues present in the crystal protein form disulfide bridges (Nickerson, K., 1980).

The free sulfhydryl content (Table 6) was determined by reacting native crystals with iodoacetic acid in the presence of 8M urea. The amount of free sulfhydryl was quantified by amino acid analysis as CM-Cys. The crystals were allowed to swell in 8M urea in order to maximize the accessibility of the free sulfhydryl groups to the reagents. The reaction was

TABLE 6

Cysteine content of the *B. thuringiensis* subsp. *kurstaki* HD-1 and *B. thuringiensis* subsp. *entomocidus* crystal protein*

Procedure or Product Obtained	*B. thuringiensis* subsp. *kurstaki* HD-1	*B. thuringiensis* subsp. *entomocidus*
cystine	8.0 ± 1.0	7.0 ± 1.5
cysteic acid	18.1 ± 1.7	15.3 ± 2.3
carboxymethylcysteine	15.9 ± 1.0	18.6 ± 0.8
free sulfhydryl	1.9 ± 1.0	1.2 ± 0.2

*moles/mole of 130 kDa protein with a standard deviation of five analysis.

allowed to proceed for 30 min or 24 h and it was found that the amount of free sulfhydryl obtained was the independent of the time of reaction. Values of 1.9 ± 0.1 and 1.2 ± 0.2 moles of free sulhydryl groups per mole of 130-kDa protein was obtained for B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus respectively.

In order to determine which of the cystines within the protoxin exist as free sulfhydryl groups, native crystals were reacted with [^{14}C]iodoacetic acid. The crystal protein was then digested with pepsin and subjected to high voltage paper electrophoresis at pH 6.5. If any of the free sulfhydryl groups existed as a unique population, then autoradiography of the chromatogram would show a distinct banding pattern. Such a pattern is not obtained, in fact, a very large number of radioactive peptides are seen on the autoradiogram Figure 6. No evidence for the specific incorporation of radioactivity into one or two particuliar groups was obtained.

Alignment of Disulfide Bridges

The method of Brown and Hartley (1966) was used to align the disulfides present in the crystal. The "fingerprinting" of cysteic acid peptides derived from disulfide bridges consists of separating cystine peptides by paper electrophoresis followed by oxidation on the paper by performic acid vapours. Electrophoresis at a right angles to

FIGURE 6

Autoradiogram of the pepsin digest of [^{14}C] carboxymethyl crystal protein after electrophoresis and exposure for 2 days. A, reaction. The reaction time was 10 min and (b) for 24 h.


A
B
O
O
−
+

the first direction produces parallel groups of cysteic acid peptides lying off the diagonal, from which the alignment of the cysteine in the native structure can be deduced.

The alignment of the peptides present in the neutral band is achieved by separation of the peptides at pH 2.1 followed by performic acid oxidation and a second electrophoresis at pH 6.5 at right angles to the pH 2.1 separation. The bulk of the peptides remain neutral and appear as a large band at the origin. Peptides that were linked by disulfide bridges will then be paired in vertical columns.

The method developed by Brown and Hartley (1966) has general applicability to proteins of molecular mass less than 40-kDa. The molar amounts of peptides that are generated from proteins of this molecular mass are sufficient for detection of off-diagonal peptides by cadmium-ninhydrin staining. Due to the high molecular mass of the B. thuringiensis crystal protein (i.e. 130-kDa) a modification to the original method was needed in order to obtain sufficient molar amounts of peptides (see methods) for detection.

The diagonal disulfide map obtained from B. thuringiensis subsp. kurstaki HD-1 is illustrated in Fig. 7A. The diagonal fingerprint has most of the cystine-containing peptides in the neutral band, with one strongly staining acidic peptide (HD1-1) as well as several fainter spots in the acidic and basic

FIGURE 7

Diagonal peptide maps of the B. thuringiensis subsp. kurstaki HD-1 crystal protein digested with pepsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

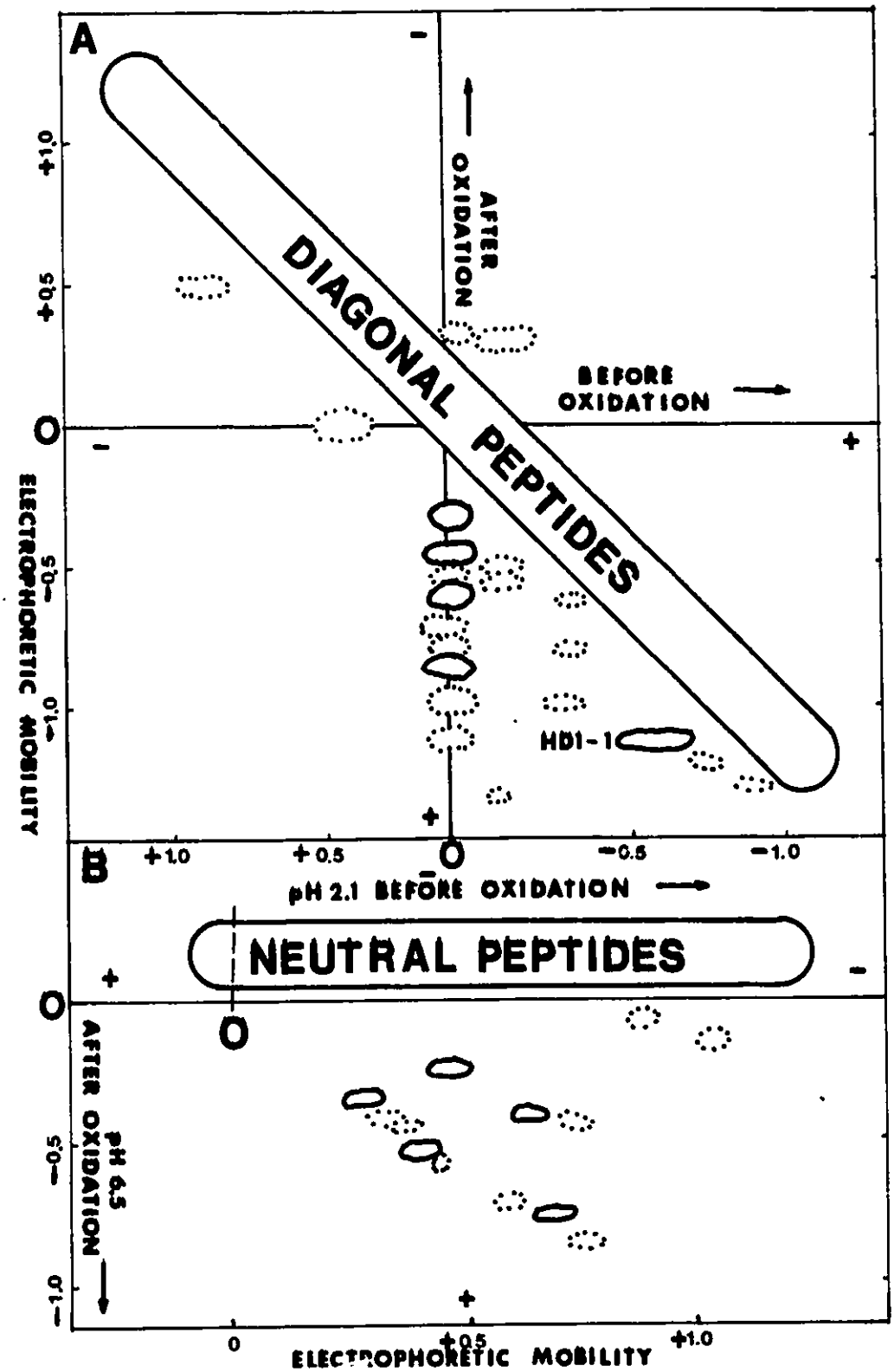
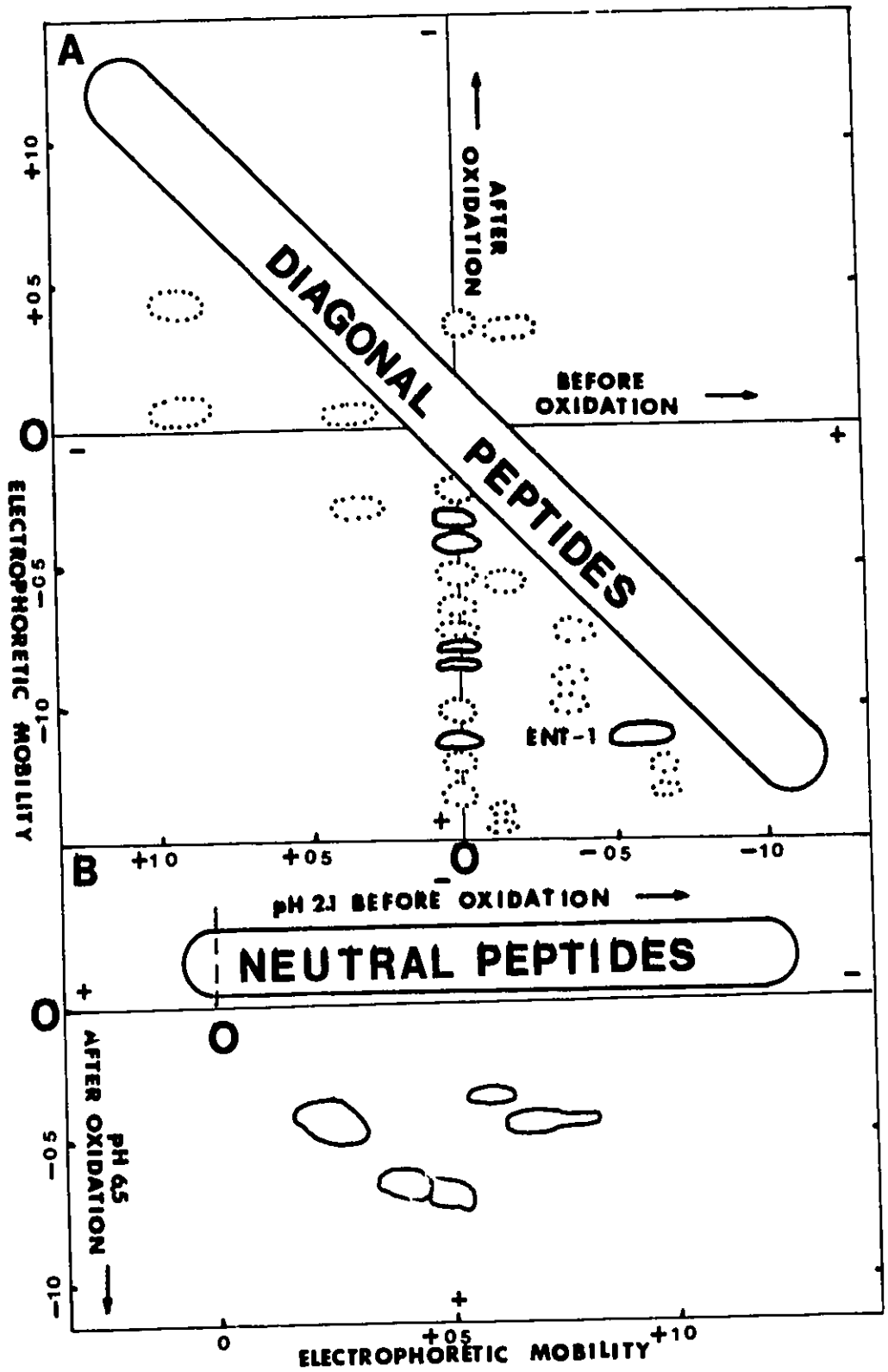

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
HD1-1
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
−0.5
−1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
AFTER OXIDATION pH 6.5
ELECTROPHORETIC MOBILITY

FIGURE 8

Diagonal peptide map of the B. thuringiensis subsp. entomocidus crystal protein digested with pepsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.


A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ENT-1
ELECTROPHORETIC MOBILITY
+10
+05
0
−05
−10
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

regions of the chromatogram. The neutral band diagonal fingerprint obtained from these crystals is illustrated in Fig. 7B. The neutral band contains several strongly staining peptides and faintly staining peptides. None of the major peptides are paired in either of these chromatograms.

The diagonal peptide map of the crystal protein of B. thuringiensis subsp. entomocidus (Fig. 8A) is very similar to that obtained for B. thuringiensis subsp. kurstaki HD-1. Again there is only one strongly staining peptide (ENT-1) with the same electrophoretic mobility as HD1-1. The neutral band fingerprint of this subsp. is given in Fig. 8B. Here, as was the case for B. thuringiensis subsp. kurstaki HD-1, none of the major peptides are paired.

The only strongly staining off-diagonal peptides, excluding the neutral band, were HD1-1 and ENT-1. Their analysis gave the following composition: Asp_3 $Cys(SO_3H)_1$ Glu_1 Gly_1 Ile_1 Leu_1 Thr_1 Val_1 (Table 7).

Diagonal peptide maps were also run on the following enzyme digests of B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus crystals: pepsin / chymotrypsin; pepsin / elastase; pepsin / thermolysin and pepsin / trypsin. The pepsin/elastase diagonal peptide map of B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus is illustrated in Figures 9 and10

TABLE 7

Composition of the off diagonal peptides HD1-1 and ENT-1 obtained from B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus

Amino Acid	Peptide HD1-1	Peptide ENT-1
Aspartic Acid	3.02 (3)	2.83 (3)
Cysteic Acid	0.80 (1)	0.95 (1)
Glutamic acid	1.25 (1)	1.26 (1)
Glycine	1.15 (1)	1.11 (1)
Isoleucine	0.89 (1)	0.82 (1)
Leucine	1.01 (1)	0.80 (1)
Threonine	0.91 (1)	0.91 (1)
Valine	0.95 (1)	1.02 (1)

*Calculated from the amino acid analysis as mole fraction of amino acid times ten.

The numbers in parenthesis correpond to the expected number of amino acid or predicted by the gene sequence between positions 831-840, 806-815 and 833-842 of the "4.5", "5.3" and "6.6" gene types (total 10 amino acids).

respectively. Apart from the neutral band, there are two major bands on each chromatogram. One of these bands has approximately the same mobility as bands HD1-1 and ENT-1 in the pepsin digest alone. The second band that appears is also acidic and is the result of the second digestion. It is not paired in either subspecies. The bulk of the cysteic acid peptides still lie in the neutral band in both cases and when the neutral band fingerprints are run, Figure 9B and 10B are obtained for B. thuringiensis subsp. kurstaki HD-1 and B. thuringiensis subsp. entomocidus. The second digestion generates more peptides which move in the neutral band as well as a different pattern of faint bands, (after pH 2.1 separation) however, none of these bands are paired.

The diagonal peptide maps of the other digests are illustrated in Figures 11,12,13,14,15,16,A along with the corresponding neutral band maps Figures 11,12,13,14,15,16,B. The results obtained in all of these multiple enzymatic digests are qualitatively the same as those seen for the pepsin or pepsin/elastase digestion. The most significant feature of all of these multiple digestions is that no pairing of cysteic acid peptides was observed in any of the diagonal maps.

FIGURE 9

Diagonal peptide map of the B. thuringiensis subsp. kurstaki HD-1 crystal protein digested with pepsin and elastase.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

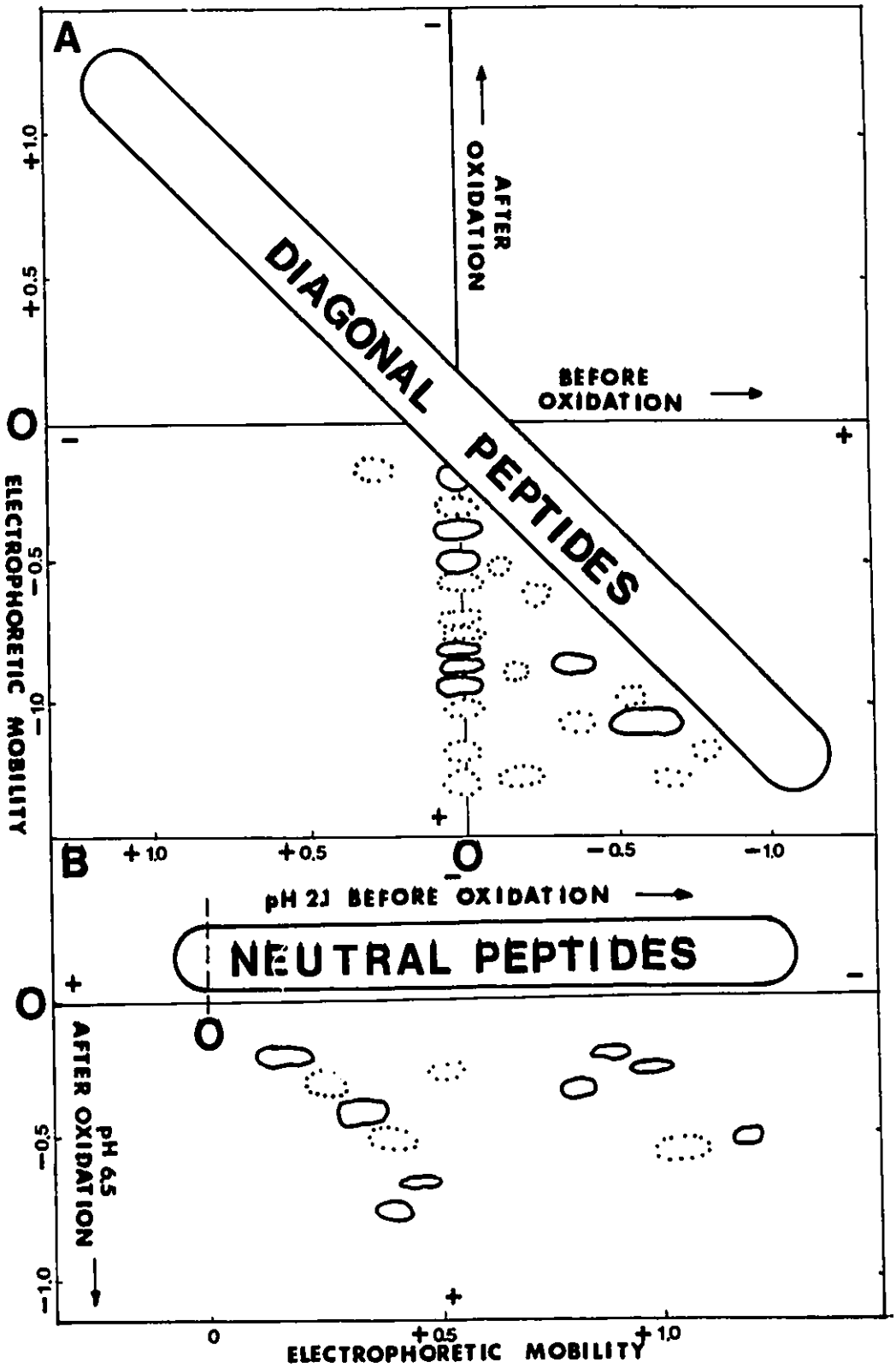

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
−0.5
−1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 10

Diagonal peptide map of the B. thuringiensis subsp. entomocidus crystal protein digested with pepsin and elastase.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

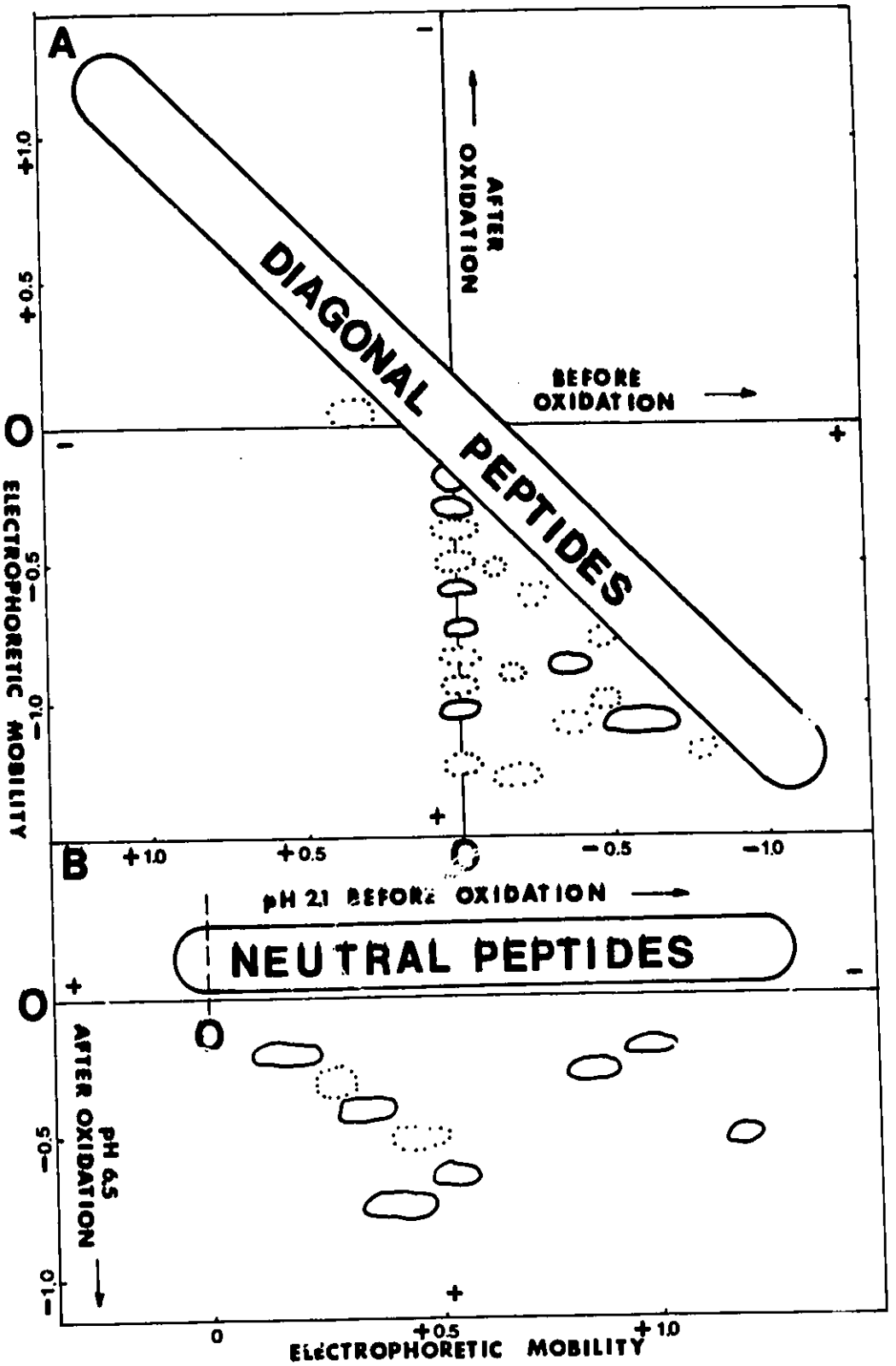

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
−0.5
−1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 11

Diagonal peptide map of the B. thuringiensis subsp. kurstaki HD-1 crystal protein digested with pepsin and trypsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

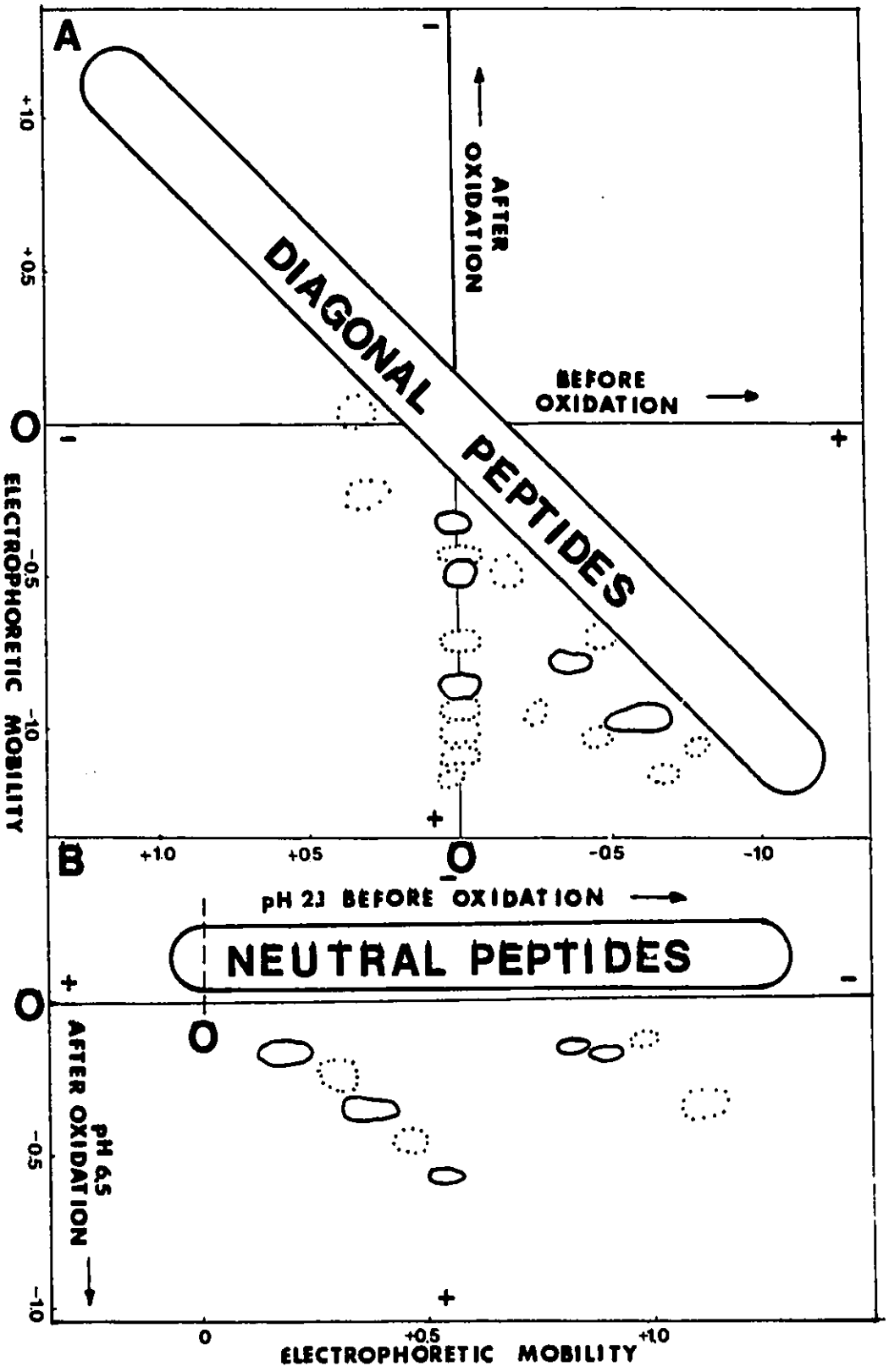

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
-0.5
-1.0
B
pH 2.3 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 12

Diagonal peptide map of the B. thuringiensis subsp. entomocidus crystal protein digested with pepsin and trypsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

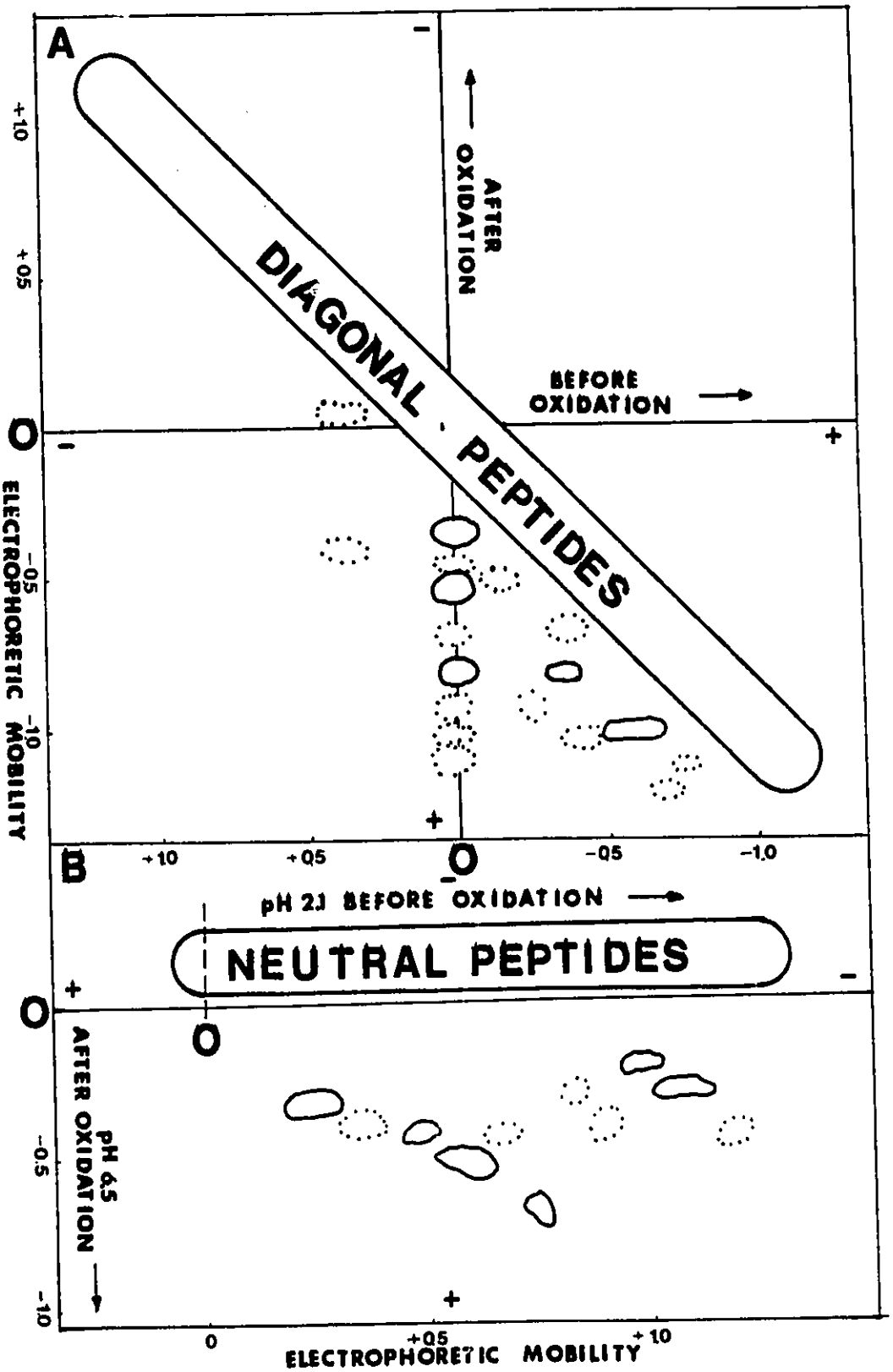

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
-0.5
-1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 13

Diagonal peptide map of the B. thuringiensis subsp. kurstaki HD-1 crystal protein digested with pepsin and α chymotrypsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

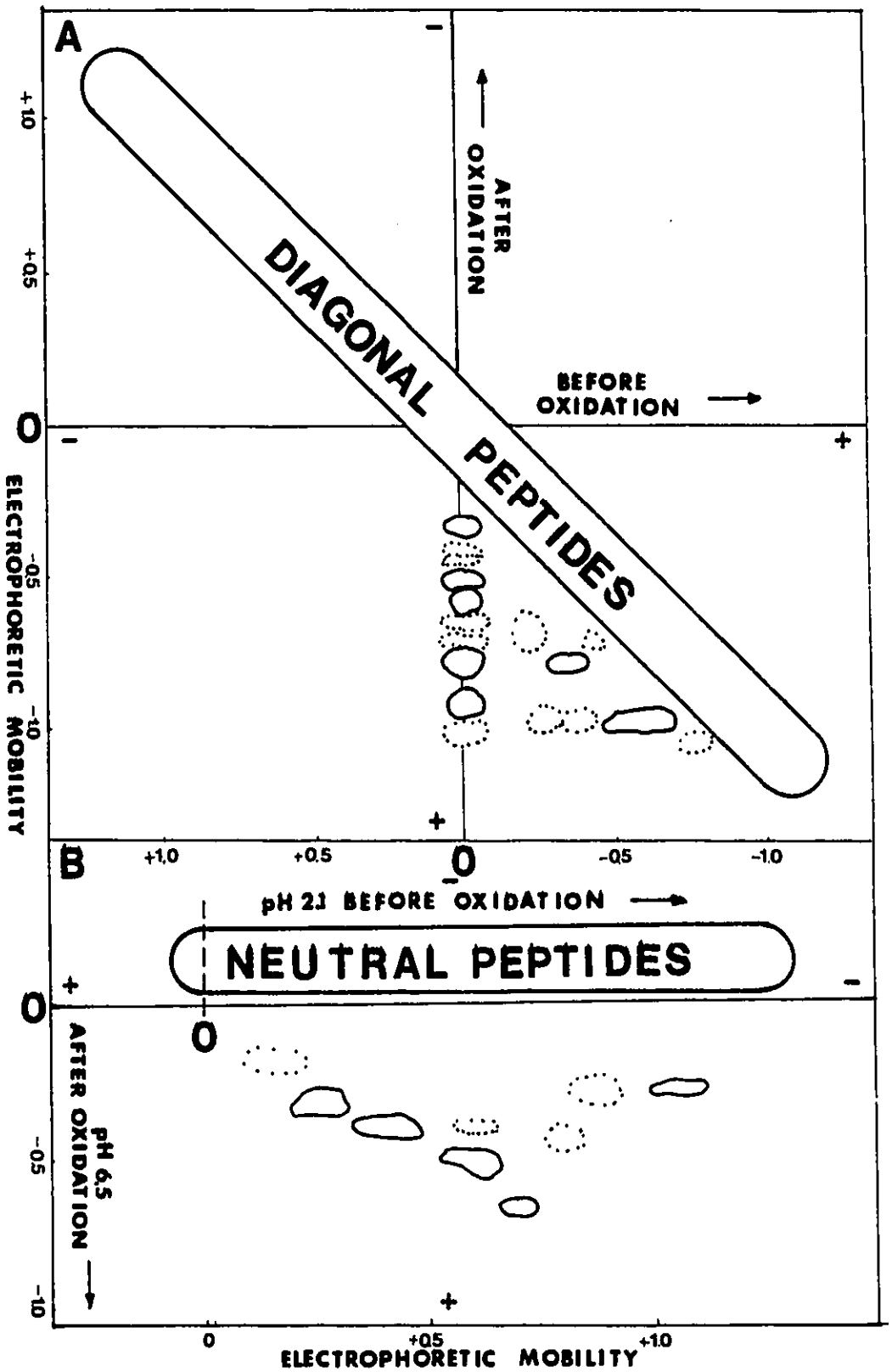

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
-0.5
-1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 14

Diagonal peptide map of the B. thuringiensis subsp. entomocidus crystal protein digested with pepsin and α chymotrypsin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

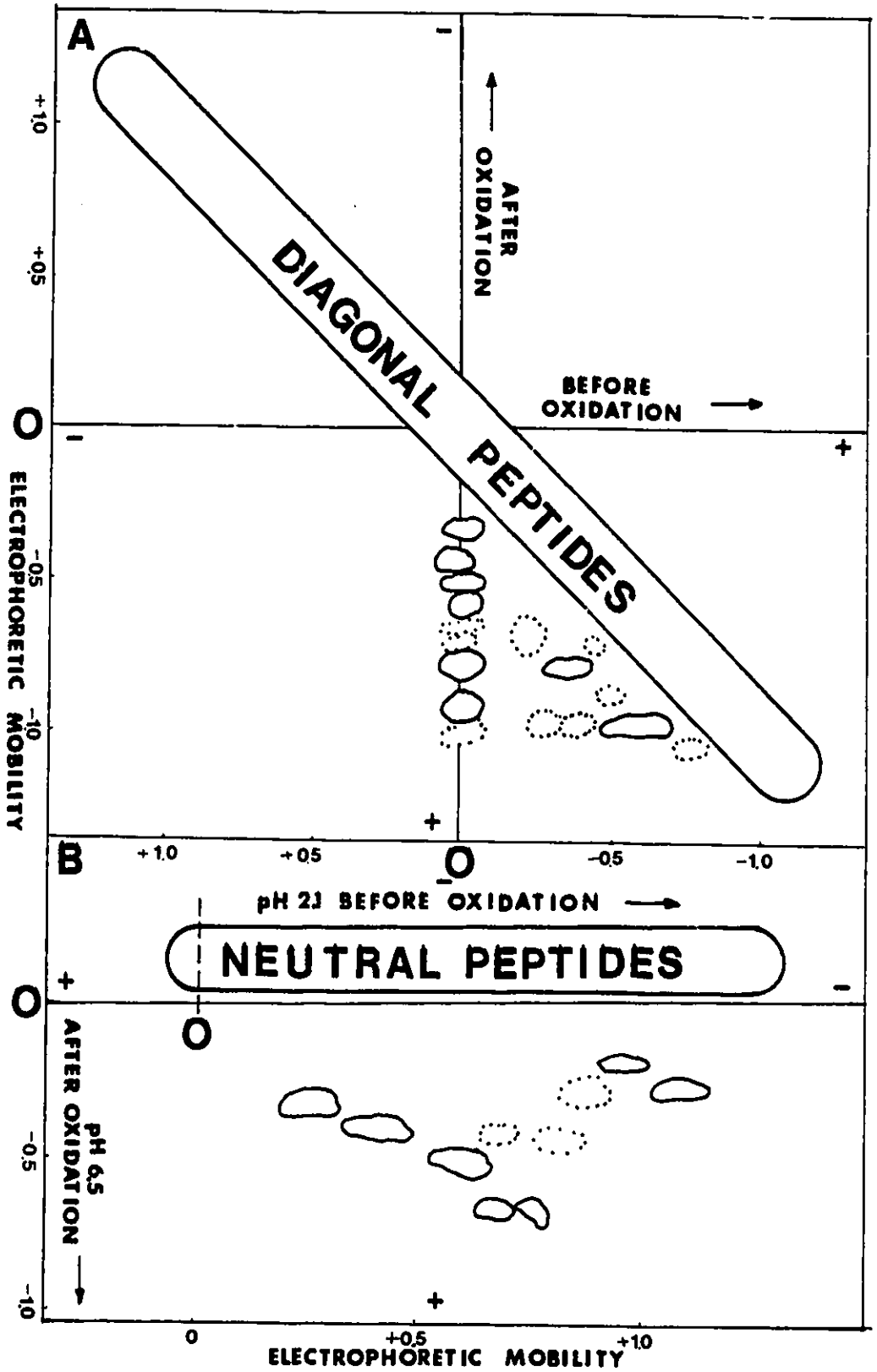

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
-0.5
-1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 15

Diagonal peptide map of the B. thuringiensis subsp. kurstaki HD-1 crystal protein digested with pepsin and thermolysin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

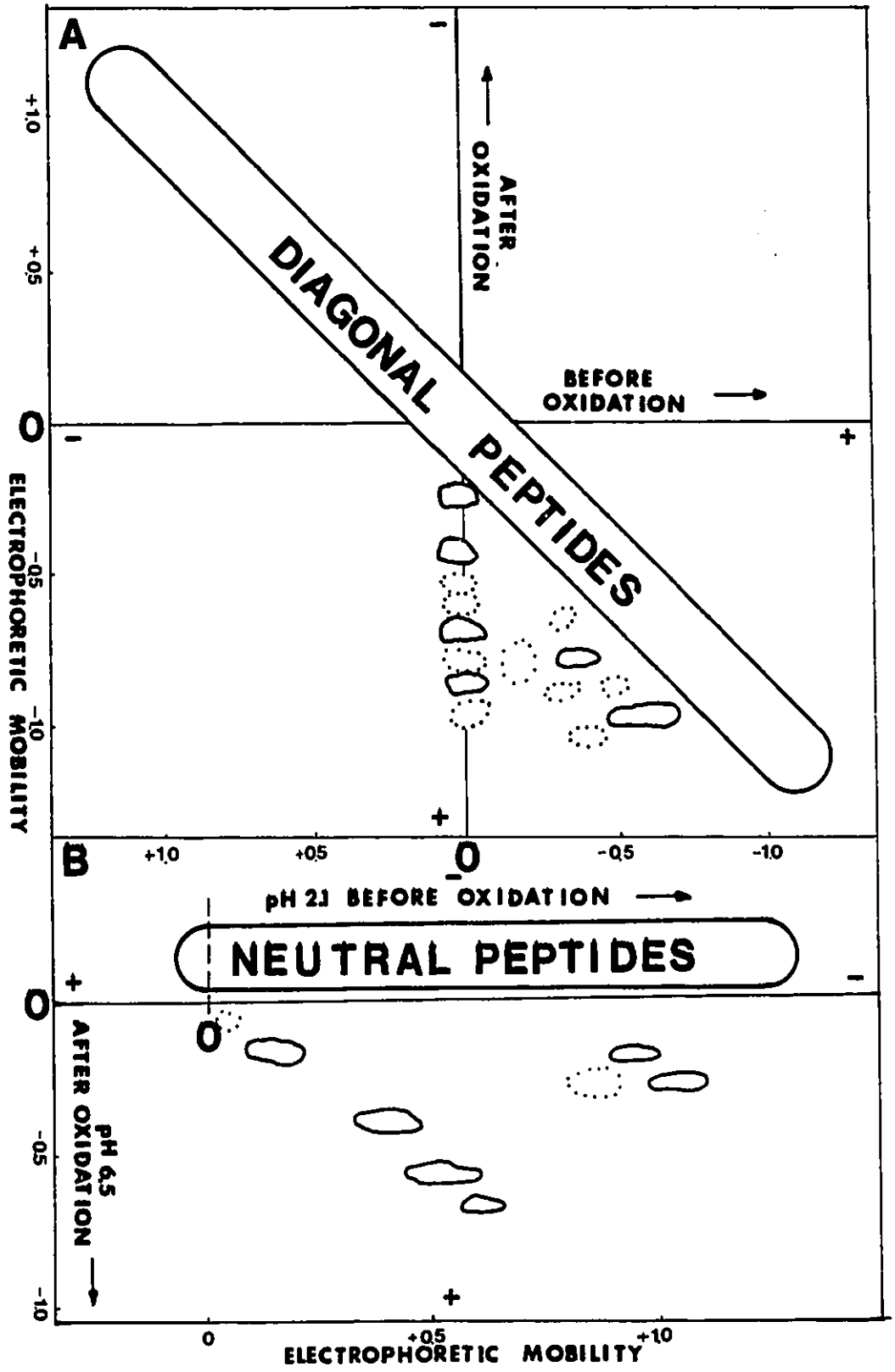
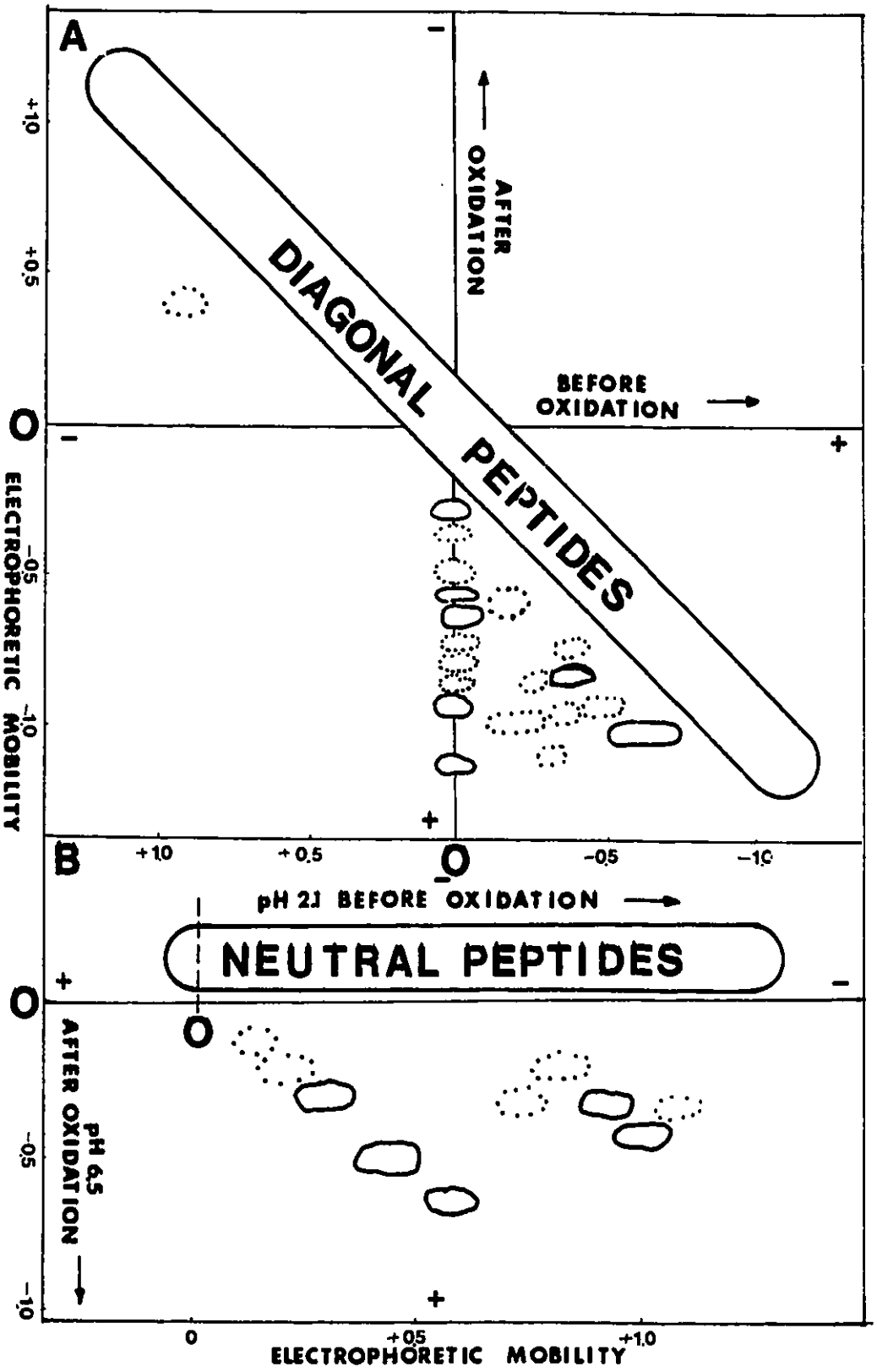

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
-0.5
-1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

FIGURE 16

Diagonal peptide map of the B. thuringiensis subsp. entomocidus crystal protein digested with pepsin and thermolysin.

Section A represents the pH 6.5 diagonal peptide map and section B is the neutral band peptide map obtained when the material is separated at pH 2.1 prior to oxidation.

The "O" marks the origin in each chromatograph. The strong staining peptides are indicated by solid lines and the faint staining peptides are in broken lines.

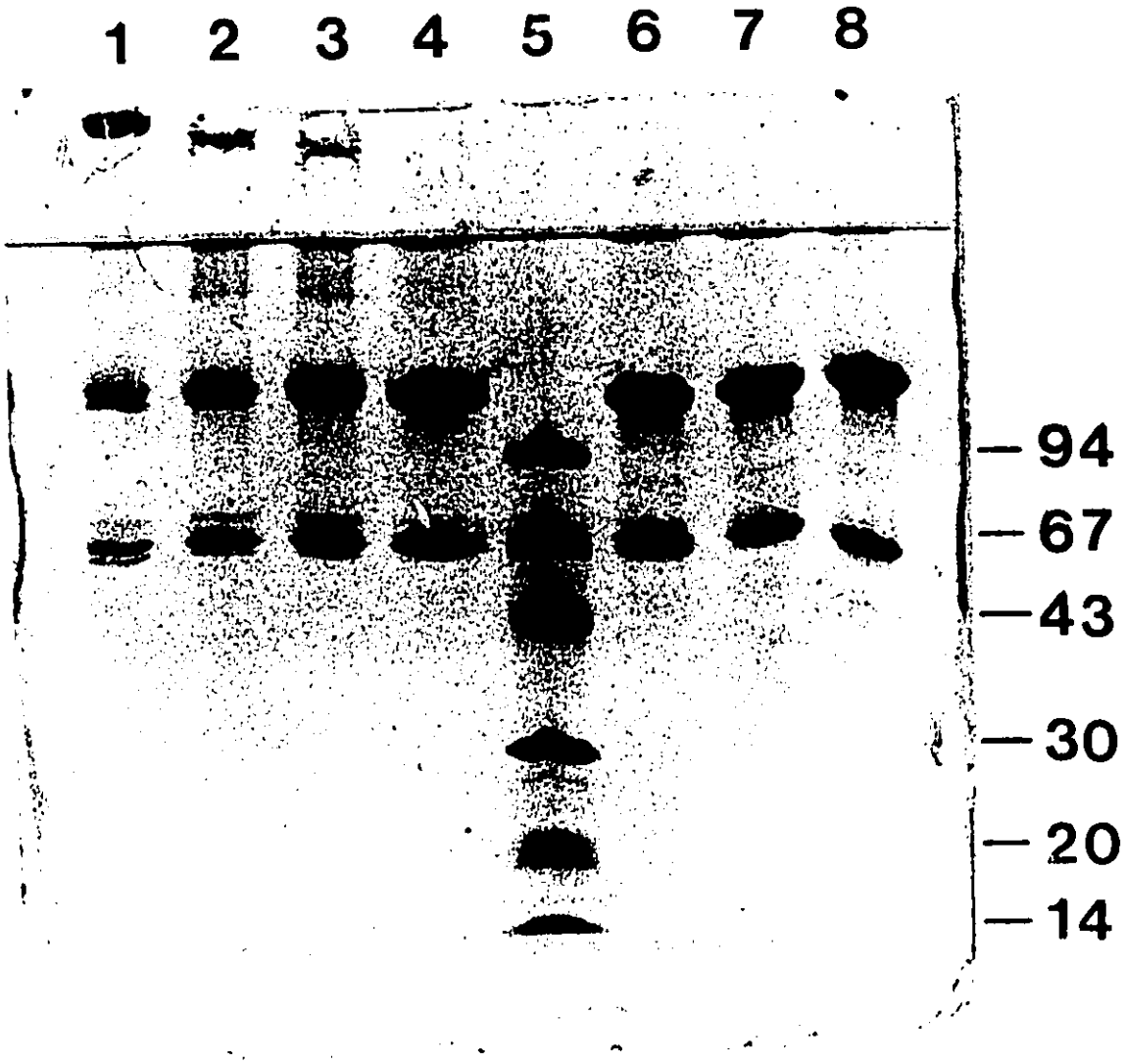

A
DIAGONAL PEPTIDES
AFTER OXIDATION
BEFORE OXIDATION
ELECTROPHORETIC MOBILITY
+1.0
+0.5
0
-0.5
-1.0
B
pH 2.1 BEFORE OXIDATION
NEUTRAL PEPTIDES
pH 6.5 AFTER OXIDATION
ELECTROPHORETIC MOBILITY

Stability of Disulfide Linkages

There is strong evidence that the insolubility of the B. thuringiensis crystal is due to the disulfide bonds which hold it together (Nickerson, K. 1980; Huber, H. et al., 1981; Sattelle, D., Haniff, C., Thomas, W.a nd Ellar, D., 1985). In order to test the lability of these bonds, crystals were treated with different amounts of 2-mercaptoethanol at pH 10.5 and run non reducing gels. The reformation of disulfide bonds was prevented by carboxymethylating any free sulfhydryl groups. The results obtained are presented in Fig. 16. In lane 1, no reducing agent is present and as can be seen, at pH 10.5, some protoxin as well as mosquito factor is solubilized. The majority of the material in this lane is still at the top of the stacking gel. From lanes 2-8 the concentration of 2-mercaptoethanol is increased and as it increases so does the amount of protoxin and mosquito factor which is solubilized. A small amount of intermediate with a molecular mass of 210, kDa is seen in lane 2 & 3. This high molecular mass intermediate as well as all the material at the top of the stacking gel is solubilized with the addition of 0.01% of 2-mercaptoethanol. The fact that some protoxin is released from the crystal at alkaline pH, in the absence of 2-mercapto-ethanol indicates that the sulfhydryl groups can be broken by the alkaline conditions.

FIGURE 17

SDS PAGE gel of B. thuringiensis subsp. kurstaki HD-1 protein crystal after incubation with various concentration of 2-mercaptoethanol (1) 0%; (2) 0.001%; (3) 0.002%; (4) 0.005%; (5) molecular weight markers; (6) 0.01%; (7) 0.02%; (8) 0.05%.


1
2
3
4
5
6
7
8
94
67
43
30
20
14

Competitive Labelling

Competitive labelling (Kaplan H. et al., 1971; Young M. and Kaplan, H. 1989) of the solubilized protoxin was carried out at pH values between 7.0 and 10.0 with alanylalanine added as an internal standard. The reactivity of the side chains of the cysteine residues of the solubilized protoxin, relative to the α-amino group of alanylalanine, was determined by reacting with a trace amount of [^{3}H]Dnp-F. Under these conditions the functional groups in the protein compete for the label, and the amount of ^{3}H-label incorporated is dependent on the reactivity of the functional group at the pH value of the labelling. In order to determine the relative reactivities of the functional groups, it is necessary to determine the incorporation of the ^{3}H-label into each group. This was achieved by adding equal amounts of S-[^{14}C]DNP-cysteine, and [^{14}C]DNP-alanylalanine to each reaction mixture, extracting the [^{3}H]/[^{14}C]DNP-alanylalanine and then hydrolyzing the mixture in 6N HCl. Each of the [^{3}H]/[^{14}C]DNP-derivatives was purified by HPLC and its ^{3}H/^{14}C-ratio quantified by scintillation counting. This ratio was corrected for the amount of cysteine present in the protoxin (Table 6).

The data were analyzed using the expression:

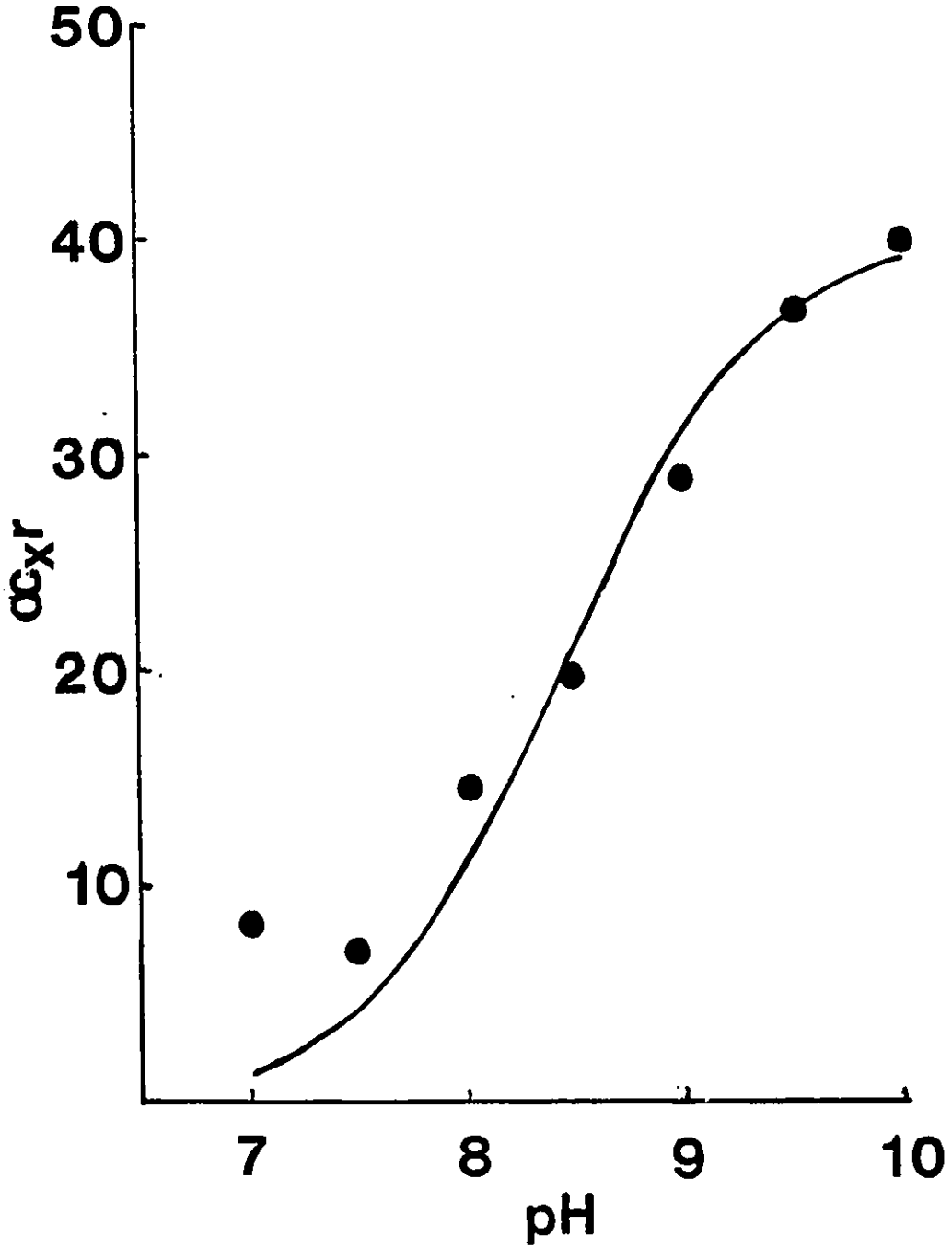
$$\alpha_x r = \alpha_s \ (^3H/^{14}C)x. \ (^3H/^{14}C)s$$

Where α_x is the degree of ionization of the functional group under study, α_s is the degree of ionization of the internal standard, alanylalanine (pK = 8.31) at 25°C, r is the pH - independent second order velocity constant for the reaction of the functional group under study relative to the amino group of alanylalanine, and $(^3H/^{14}C)x$ and $(^3H/^{14}C)s$ are the radioactivity ratios determined by scintillation counting for the functional group and alanylalanine, respectively. A plot of $\alpha_x r$ versus pH will give a titration curve with pK = pH at the inflection point and a limiting value of $\alpha_x r = r$ if the groups under study titrate normally over the entire pH employed.

The reactivity data obtained over the pH range 7-10 for the cysteine side-chains of the protoxin from Bacillus thuringiensis subsp. kurstaki HD-1 is shown in Figure 18. The data for the cysteine residues fit a theoretical titration curve with a pK of 8.45 ± 0.13 and reactivity (r) 39.6 ± 2.7 times that of the amino group of alanylalanine. The pK value of the sulfhydryl group is close to that of model compounds (Shaked, Z., Szajewski, R. and Whiteside, G. 1980), and their reactivity appears to be close to that expected for sulfhydryl groups based on the relative nucleophilicities of amino and

FIGURE 18

Reactivity-pH profile for the reaction of the sulfhydryl groups of the solubilized protoxin from B. thuringiensis subsp. kurstaki HD-1 with Dnp-F. The solid line is a theoretical titration curve with pK = 8.45 and r = 39.6. Solvent conditions were 0.1M KCl - 5mM N-ethylmorpholine - 5mM sodium borate at 25°C.


50
40
30
20
10
αc_{XI}
7
8
9
10
pH

sulfhydryl groups (Glazer, 1976).

The exposure of the sulfhydryl groups in the solubilized native protoxin was quantified by reacting with iodoacetate and iodoacetamide in the absence of any denaturant. The following CM-Cys content was obtained on amino acid analysis: a) Iodoacetate; 16.5 moles CM-Cys/mole protoxin and b) iodoacetamide; 16.6 moles CM-Cys/mole protoxin.

DISCUSSION

In recent years, the use of commercial bioinsecticides has increased dramatically. The overall objective of this work was to study the structure function relationship of one of these bioinsecticides, the B. thuringiensis crystal protein (δ- endotoxins, protoxin). The following experimentation was carried out; 1) characterization of the proteolytic activity associated with the crystal protein, 2) quantification of the photoinactivation of the crystal protein and 3) the determination of the alignment of the disulfide bridges in the protein crystal.

Proteolytic Activity of Protein Crystal

The results that were obtained from column chromatography show that crystal purified by Renografin-76 step gradient (Fast, P., 1972) did not provide proteinase free crystals. The elution profile of these crystals obtained from a sephacryl S-300 gel permeation column showed that all high molecular mass material was broken down into small peptides over a period of one month when stored at 4°C in water. The lack or intermediate degradation products at any time suggests that after an initial cleavage, the products were further degradated very rapidly into small peptides. This is consistent with the results of Fast, P. and Martin, W. (1980)

which state that the crystal is made up of small polypeptides of molecular mass of approximately 1 kDa. It is suprising that the proteinase resistant core or "ultimate toxin" (Huber, H. and Lüthy, P., 1981 and Aronson, A. et al., 1986) is not seen at any time. The absence of mosquito factor (molecular mass 66 kDa) can be accounted for by the fact that it has a high affinity for the column matrix (Yamamolo, T. and McLaughlin, R., 1981). Chestukhina, C. et al., (1980) have shown that proteinases are produced in large amounts during sporulation. Bulla, L. Jr. et al., (1977) have shown that various types of proteolytic activity are present during sporulation. This suggests that the labile nature of the solubilized protoxin is due to the presence of various proteinases which are present in trace amounts in the crystal protein preparation.

The possibility of extensive proteolysis during crystal solubilization was also investigated. Crystals are customarily solubilized by using reducing agents such as 2-mercaptoethanol or dithioerythritol at alkaline pH's (Yamamoto, T. and McLaughlin, R. 1981). Under these conditions, even in the presence of 8M urea, proteolysis of the crystal protein takes place as seen by the multiple N-termini present (Fig. 3A). When conditions are such that proteolysis is prevented (pH 3.0, 8M urea) only one N-terminus is obtained in our analysis (Fig. 3B). At this pH, any contaminating proteinases are

completely denatured by the combination of low pH and urea and hence are completely inactive. When the pH is raised and the crystal solubilized with 2-mercaptoethanol, no proteolysis of the crystal takes place as demonstrated by the single N-terminus present.

These results point out the need for a better method of purifying proteinase free protein crystal. This was achieved by Renografin-76 step gradient and extensive washings with water to remove spores and proteinases (Carey, P. et al., 1986). This method provides crystal with a crystal:spore ratio in excess of 1000:1, which are proteinase free as demonstrated by the lack of proteolytic activity towards denatured [^{14}C]methylated hemoglobin. These highly purified crystals were used in all subsequent experiments.

The results obtained from this series of experiments help explain the confusion which existed in the early literature about the size of the crystal protein. It is now generally accepted on the basis of gene sequence analysis and molecular mass determinations that the crystal protein has a molecular mass in the range of 130-140 kDa. The early reports of multiple protein components in lepidopteran-specific crystals as well as their varying molecular masses can be explained by the action of proteinases adsorbed on the surface of the crystal.

Solar Inactivation of the Protein Crystal

The inactivation of proteins can occur through oxidation. The oxidation of amino acid side chains is a widespread phenomenon (Volkin, D. and Klibanov, A., 1988). Aromatic sidechains, especially histidine, tyrosine and tryptophan as well as methionine, cysteine and cystine are targets of the oxidation process (Grossweiner, L., 1984 and Volkin, D. and Klibanov, A., 1988). Acid hydrolysis is routinely used to determine the amino acid composition of peptides and proteins. This method yields quantitative results for all amino acids except tryptophan residues which are destroyed by this procedure (Darbre, A., 1986). As such, a separate hydrolysis using methanesulfonic acid (Simpson, R., Newberger, M. and Liu, T., 1976) was used to quantify tryptophan. Histidine, tyrosine and methionine contents of the crystal protein were quantified by acid hydrolysis in 6N HCl.

The main finding of this investigation is that the extent of destruction of amino acid side-chains seen in all three crystal preparations is almost identical. We also found that the rapid loss of biological activity did not correlate with the rate of destruction of the amino acid side-chains which were investigated.

The loss in toxicity of B. thuringiensis crystal on exposure to sunlight has been reported by many authors (Beegle, C., Dulmage, H., Wolfenbarger, D. and Martinez, E., 1981; Hüber, H. and Lüthy, P. 1981). Raman spectroscopy showed that there was a decrease in the tryptophan features in the spectrum when the crystal protein was exposed to "sunlight" in a solar simulator (Carey, P. et al., 1986 and Pozsgay, M. et al., 1987). Since the number of tryptophans destroyed and the rate at which their destruction occured were not determined, experimentation aimed at quantifying this destruction was carried out. The data presented in this thesis are the first quantitative results on the effect of "sunlight" on the amino acid side-chains in B. thuringiensis crystal protein. This was made possible by the use of a solar simulator which permitted the controled exposure of the crystal to "sunlight" (Carey, P. et al., 1986 and Pozsgay, M. et al., 1987).

Acid hydrolysis, which is routinely used to quantify the amino acid content of proteins, results in the destruction of tryptophan. Tryptophan destruction in HCl may be limited by the addition of thioglycolic acid. However, the use of sulfonic acids for hydrolysis has been widely used since they are non oxidizing acids which give quantitative recoveries for tryptophan (Simpson, R., 1976). Methanesulfonic acid

hydrolysis was chosen for practical as well as technical reasons. The accuracy of this method was verified by using standard proteins. The results obtained for tryptophan analysis of bovine α-chymotrypsin (4 tryptophan molecules per mole) and chicken lysozyme (6 tryptophan molecules per mole) demonstrated that this method could be used to give quantitative results for tryptophan analysis.

The results obtained for B. thuringiensis subsp. kurstaki HD-1 show a total loss of 40-50% of the total tryptophan present in the crystal after 32-36 h of irradiation with no further loss thereafter. This represents a loss of 8-9 residues per molecule. A loss of 20% of the histidine is seen after 50-60 h of irradiation. The rate of histidine destruction is less than that of tryptophan destruction and represents the destruction of 4-5 imidazole side-chains. The destruction of the tyrosine side-chain occurs at a very minimal rate. A decrease of 5% is seen in the tyrosine content after 60h of irradiation. The methionine content decreases by 10% to 15%. This corresponds to a loss of 1-2 residues of methionine per molecule of protein.

Multiple products are known to be generated by the oxidation of tryptophan, histidine, and tyrosine side-chains making their identification and quantification very difficult. Tryptophan is believed to be converted to kynurenine or

formylkynurenine (Pileni, M-P., Santus, R. and Land, E., 1978). These compounds were not detected by amino acid analysis however, they are known to break down into many oxidation products. Imidazole side chains are broken down to aspartic acid and urea via many intermediate compounds (Means, G. and Feeney, R., 1972). Tyrosine side chains are also sensitive to oxidation, however the products generated are not well characterized. The oxidation of methionine is a well characterized process. Methionine is first oxidized to methionine sulfoxide and then to methionine sulfone. Methionine sulfoxide is converted back to methionine upon acid hydrolysis and as such, cannot be quantified directly. No methionine sulfone was detected in these analysis indicating that in all probability no methionine sulfoxide was formed.

The rate at which biological activity is lost is greater than the rate of destruction of any of the amino acid side-chains studies. After 8h of irradiation the toxic activity of the crystal is lost. At this point 20% of the tryptophan and 5-6% of the histidine are destroyed. The toxicity results are consistant with those reported by Morris, O., 1983 who reported that a "marked loss in toxicity" occured when this crystal was exposed to direct sunlight for 4.5 h.

The damage caused by sunlight inactivation in this protein is unusual. A pure protein containing no prothetic group does not absorb light above 290 nm, and sunlight reaching the earth's surface has no intensity below 300 nm, therefore it is unlikely that the damage seen is due to the direct absorption of a photon by individual amino acid side-chains. Another mechanism is needed to account for the destruction of indole and imidazole side-chain. "Photodynamic action" can be used to explain the destruction seen. In this mechanism, a moiety, other than an amino acid side-chain, absorbs light and transfers the absorbed energy to a neighboring oxygen molecule generating singlet oxygen. This is a very reactive species which would cause widespread damage throughout the protein with indole and imidazole being favoured targets (Grosswiener, L., 1976; Grosswiener, L., 1984).

The chromophore involved in this process has not been isolated and characterized but various authors have speculated about its origin. There are various sources from which these chromophores can arise. The products of tryptophan oxidation are known to be photosynthesizing agents (Pileni, M-P., et al., 1987). Another possibility is that the chromophore is made by the bacteria and incorporated into the crystal. Harms, R. et al., (1986) isolated coproporphyrin which were secreted

during every stage of the bacterium's life cycle. These compounds can act as photosynthesizers however, they absorb at wavelengths which do not cause a loss in toxicity (Morris, O., 1983 and Carey, P. et al., 1986). A final possibility is that "chromophores" are absorbed from the growth medium. These chromophores would be adsorbed onto the surface of the crystal during their harvesting procedure.

The last hypothesis was tested by lysing the bacteria in water instead of growth medium. The loss of toxicity of the water-lysed sample was markedly reduced upon irradiation of this sample (unpublished results).

Unfortunately, most work aimed at increasing the field life of insecticides formulated with B. thuringiensis has focused on crystal-spore mixtures. The materials used as protectants include molasses, yeast extract, proteins, protein hydrolysate and nucleic acids (Auffray, Y. and Boutinbannes, P., 1987; Andrews, R. et al., 1987). Commercially registered photoprotectants have also been used but their effects were marginal at best (Andrews, R. et al., 1987).

It appears from these results that the loss of biological activity is a more complex phenomenon than originally believed. The inactivation of the crystal involves the destruction of amino acid side chains but the destruction demonstrated here does not account for the total loss.

Characterization of the Cysteine Residues

The third investigation in this thesis deals with the characterization of the cysteine residues which are involved in holding the individual protein molecules in the crystalline array. Three series of experiments aimed at identifying and characterizing specific aspects of the arrangement of the sulfhydryl groups were carried out. The first was intended to quantify the cystine/cysteine content of the crystal protein. At the same time the amount of free sulfhydryl groups was quantified. This was achieved by carboxymethylation of the crystal, and quantification of CM-Cys after acid hydrolysis. The second series of experiments was aimed at determining the arrangement of the cysteine residues in disulfide bridges. Here, the diagonal electrophoresis technique of Brown and Hartley (1966) was used to align the disulfide bridges within the protein crystal. The third series of experiments dealt with the lability of the disulfide bonds and application of the method of competitive labelling to determine the exposure of the cysteine residues in the solubilized crystal protein.

Cystine/Cyteine and Free Sulfhydryl

The subspecies of *B. thuringiensis* used throughout this study are subsp. *kurstaki* HD-1 and subsp. *entomocidus*. The former subspecies forms the basis of many of the commercial insecticidal formulations available in North America (Andrews,

R. Jr. et al., 1987) and as such, has been the subject of many investigations. It has been found to contain three genes which code for the 130 kDa protoxin (P1) (Andrews, R. Jr. et al., 1987 and Brousseau, R. and Masson, L., 1988). The genes predict 17, 13 and 16 cysteine residues per molecule for the "4.5", "5.3" and "6.6" kb-gene types respectively. In addition to these three genes, B. thuringiensis subsp. kurstaki HD-1 contains a gene coding for the mosquito factor (P2) of molecular mass 65-66 kDa. This gene was sequenced by Donovan, W. et al., 1988 and predicts 4 cysteine residues per molecule.

The expression of genes in sporulating bacteria is an area of extensive research. The factors which control the expression of the protoxin gene in B. thuringiensis remain unclear (Andrews, R. Jr. et al., 1987). Since this subspecies contains more than one gene coding for crystal proteins, there is a possibility that these genes are being differentially expressed. Recently Dr. L. Masson has shown that all three homologous genes are being expressed in B. thuringiensis subsp. kurstaki HD-1 (Personal communication) with the 5.3 and 6.6 kb-gene being the major components. By studying HPLC peptide maps of trypsin-digested crystal proteins of B. thuringiensis subsp. kurstaki HD-1 and HD-262 as well as by comparing them to peptide maps obtained from individual gene products, Yamamoto, T., Ehman, T., Gonzalez, J. Jr. and

Carlton, B. (1988) concluded that B. thuringiensis subsp. kurstaki HD-1 crystals contained equal amounts of the "4.5" and "5.3" kb-gene types. These authors report that the mosquito factor produced by HD-1 is a minor component of the crystal. The fact that our quantification results for the cysteine content are very close to the values predicted by the gene nucleotide sequence for the P1 protein agrees with the conclusion that P2 is a minor component of the crystal.

In the present study total cysteine content of the protein crystal was determined by performic acid oxidation which will convert all cystine and cysteine to cysteic acid. The total number of reducible cysteine residues was determined by reduction and carboxymethylation. The results obtained for both subspecies are very similar and give an average of 16-18 moles of cysteine per mole of protoxin. The amount of CM-Cys obtained after 30 min and after 24 h of reaction indicates that in the solubilized protoxin, the sulfhydryl groups were available to the iodoacetate. Equivalent amounts of cysteic acid and CM-Cys were obtained which indicates that the amount of reducing agent used to solubilize the crystal protein was sufficient to reduce all the cysteines.

The total cystine content was quantified directly by amino acid analysis after hydrochloric acid hydrolysis of the protein. An average of 7-8 cystine residues per molecule of

protoxin in both crystal types was obtained. Within the experimental error, this value accounts for all the cysteine residues present, confirming Nickerson's, K. (1980) earlier deduction that the majority of cysteine residues are involved in disulfide bridges.

The cysteine content obtained for the crystal proteins from both crystal types are consistent with the gene-deduced value of 17 and 16 cysteines for the "4.5" or "6.6" kb-gene types. Our results therefore indicate that one or both of these genes are major components of the crystal protein.

Working with B. thuringiensis subsp. thuringiensis (a lepidopteran-specific subspecies), Dastidar, G. and Nickerson, K. (1979) deduced that all sulfhydryl groups were present in the form of disulfide bridges. Couche, M. et al., (1987) working with the dipteran-specific subspecies B. thuringiensis subsp. israelensis found that only 60% of the sulfhydryl groups were in the form of disulfide bonds at pH 8.0.

In order to quantify the amount free sulfhydryl, the crystals were reacted with iodoacetic acid in the presence of 8M urea at pH 8.6. The urea was used to swell the crystal and maximize the accessibility of the crystal's interior to the reagent. After acid hydrolysis of the protein, the number of free sulfhydryl groups was quantified by amino acid analysis as CM-Cys. We find approximately 2 mole of free sulfhydryl per

monitored by amino acid analysis, and no decrease was observed for the methionine, histidine and lysine residues after labelling under the conditions described. We therefore conclude that the many radioactive peptides observed are due to the radioactive incorporation of the label into a large number of different cysteine residues.

The present results for the free sulfhydryl content of the crystal are in contrast to those obtained by Dastidar, G. and Nickerson, K. (1979) and by Nickerson, K. (1980), who worked with B. thuringiensis subsp. thuringiensis (a lepidopteran-specific subspecies). They reported finding no free sulfhydryl groups in a colorimetric determination using Ellmans reagent. However, these workers boiled their samples prior to their colorimetric determination and therefore probably oxidized any free sulhydryl group. Their results also differ from those of Couche, M. et al., (1987) who worked with B. thuringiensis subsp. israelensis (a diperan-specific subspecies), and who reported that 40% of the cysteine existed as free sulfhydryls in these crystals. B. thuringiensis subsp. israelensis produces complex crystals which are composed of various proteins ranging from 25 to 140 kDa (Brousseau, R. and Masson, L. 1988). The homology at the gene level between the 130 kDa component of this dipteran-specific crystal and that of the lepidopteran-specific crystal that we are using is low

(Brousseau, R. and Masson, L., 1988). The homology between the protoxin from B. thuringiensis subsp. kurstaki HD-1 and the other gene products present in the B. thuringiensis subsp. israelensis crystal is also low (Brousseau, R. and Masson, L., 1988), and as such a comparison between them may not accurately represent differences in disulfide arrangement but may reflect the heterogeneous nature of the crystal.

Alignment of Disulfide Bridges

There are 2,027,025 ways in which 16 cysteine residues can form 8 disulfide bridges (Chelis, G. and Yon, J., 1982). One strategy for the alignment of disulfide bridges is to isolate all peptide fragments after enzymatic digestion, followed by amino acid analysis to determine which fragments contain cystine. These fragments are further purified, the cystine broken and the individual peptides which made up the disulfide bonds characterized. Recently, the combination HPLC and amino acid analysis were used to isolate and identify cystine-containing peptides in human glucocorticoid receptors (Silva, C. and Cidlowski, J., 1989) in inhibitors from bitter gourd seeds (Hara, S., Makino, J. and Ikenaka, T., 1989), and in transducins (Wessling-Resnick, M. and Johnson, G., 1989). This method becomes very laborious and cumbersome as the number of proteolytic fragments increases. The B. thuringiensis crystal is an array of protoxin molecules each

containing an average of 16-18 cysteine residues per mole. These molecules are linked together producing a crystal with a molecular mass in the millions of daltons. The application of this technique then becomes a formidable undertaking as the number of cystine containing peptides can be very large.

Another approach for aligning disulfide bridges is the elegant diagonal electrophoresis method originally developed by Brown and Hartley (1966). This method is based on the fact that when a cystine peptide is oxidized by performic acid it will give rise to two cysteic acid peptides with different electrophoretic mobilities than the cystine peptide from which they originated.

The method as originally described by Brown and Hartley is not applicable to proteins of molecular mass greater than 40 kDa. This is due to the fact that an insufficient amount of the protein digest (max. 1 mg/cm) can be applied to the paper for detection of off-diagonal peptides. One solution to this problem was used in the case of α_2-macroglobulin, (molecular mass 725 kDa) where the protein was fragmented using cyananogen bromide. The individual fragments were isolated and subjected to the diagonal electrophoresis proceedure. This permitted sufficient molar amounts of peptides to be applied to the paper so that off-diagonal peptides could be detected (Sottrup-Jensen, L., Lonblad, P.,

Jones, C. and Stepanik, T., 1984; Sottrup-Jensen, L., Stepanik, T., Jones, C., Conblad, P., Kristensen. T. and Wierzbicki, D., 1984; Jensen, P. and Sottrup-Jensen, L., 1986). In the case of the protein crystal it is several orders of magnitude larger than α_2-macroglobulin complex, has limited solubility and an unfavourable distribution of methionine residues. As such, the procedure used to overcome the problem of detection in the case of α_2-macroglobulin is not applicable. A modification to the original diagonal proceedure was developed so that it could be applied to large proteins. In the first dimension, all peptides from the total digest are spotted out in a single band. After electrophoresis in the first dimension the peptide are spread out over the length of the chromatogram. They therefore can be concentrated into smaller bands without overloading the paper. In the original procedure, a 3 cm guide-strip is removed for performic acid oxidation, however, due to the high molecular mass of the crystal protein, the entire 10 cm band was used. The 10-cm strip was sewn onto a larger sheet and buffer was applied to each side, so that it concentrated the peptides into the centre of the strip. By concentrating the material in this way, sufficient amounts of material were present in a 3 cm strip for the detection of off-diagonal peptides after electrophoresis in the second dimension. This modification is

far less laborious and more general in its applicability than that described by Sottrup-Jensen, L. (1986-1984) for α_2-macroglobulin.

The interpretation of the data using this technique is very simple as peptides which were linked in disulfide bridges will lie off the diagonal line in vertical pairs. The cysteines residues of these two peptide were therefore linked in a disulfide bond in the native protein. In most cases the peptide lies off the diagonal line in pairs. In the case of the crystal protein, an unusual result is obtained in that all off-diagonal peptides are unpaired.

Unpaired cysteic acid peptides can arise with the diagonal method in three instances: (i) a single polypeptide with an intrachain disulfide; (ii) two identical cysteine peptides joined by an interchain disulfide bond; (iii) intrachain disulfide bonds between repeating sequences. As the B. thuringiensis crystal protein has been shown by gene sequencing not to contain any repeating sequences, the last possibility can be eliminated. The former two possibilities may be distinguished by treating the pepsin digest with another enzyme in order to cleave peptides with an intrachain disulfide and observing if any paired peptides appear on the diagonal maps. The fact that, with crystals from two different subspecies of B. thuringiensis, no pairing of the major

peptides was observed with any enzyme digest viz., pepsin, pepsin/chymotrypsin, pepsin/elastase, pepsin/thermolysin and pepsin/trypsin, strongly suggests that the vast majority of the disulfide brdiges which link the polypeptide chains in the crystal protein are between the same cysteine residues on each chain. That is, the cysteine residues form predominantly symmetrical interchain bonds in the protein crystal.

In all the peptide maps, a strong band appeared at position HD1-1 and ENT-1. The highly acidic nature of this peptide makes it a likely candidate for an internal disulfide which was not cleaved by the enzymes used. The amino acid analysis gave one cysteic acid residue per mole of peptide. The acidic nature of these peptides was accounted for by the high content of glutamic and aspartic acid as seen on amino acid analysis (Table 7). This peptide corresponded to the cysteine residue originating from positions 835, 810 and 837 of the "4.5", "5.3" and "6.6" kb-gene types of B. thuringiensis subsp. kurstaki HD-1 and the corresponding positions on the "4.5" kb-gene type of B. thuringiensis subsp. entomocidus.

In a few cases pairing of some of the faint spots (e.g. Figure 6a) is observed. However, these are minor products which vary with each chromatogram and could arise after digestion from: 1) oxidation of trace free sulfhydryl present,

2) a small amount of non-symmetrical bridges present in the crystal, or 3) due to disulfide interchange. We cannot absolutely rule out the possibility that they may be derived from an intrachain disulfide which, because of its particular structure, is generated in low yield. Other than this observation, no evidence was obtained for the existence of any intrachain disulfide bridges in the crystal protein.

Stability of disulfide linkages

Creighton, T. (1986) has demonstrated on the basis of kinetic and thermodynamic considerations that intrachain disulfides have a much greater stability than interchain disulfides. In order to test whether there are two such classes of disulfide linkages within the crystal protein, varying amounts of 2-mercaptoethanol were added to the crystals from kurstaki HD-1 subspecies. This was followed by reaction with iodoacetate to prevent reformation of disulfide linkages, and then the protein was run on non-reducing SDS gels. Some 130 kDa protoxin protein and 65 kDa mosquitocidal protein (Yamamoto, T. and McLaughlin, R., 1981) are solubilized at pH 10.5 in the absence of 2-mercaptoethanol (Figure 16, lane 1) but the bulk of the protein remains at the top of the stacking gel. As the 2-mercaptoethanol concentration is increased, more of the crystal protein goes into the gel, and a small amount of an intermediate with

molecular mass 210 kDa is observed (Figure 16, lanes 2 and 3). The entire crystal protein is solubilized with only 0.01% v/v (ca. 1mM) 2-mercaptoethanol (Figure 16, lane 6) indicating a very high lability of the disulfide linkages. Couche, G. et al. (1988) observed a similar effect with dithioerythritol and the protein crystal from B. thuringiensis subsp. israelensis. The free sulfhydryl observed at pH 8.6 (Table 1) most likely arises from the alkaline lability of the interchain disulfide bridges in the protein crystal.

The protoxin would be expected to have a higher mobility in non-reducing SDS gels than in reducing SDS gels, if any intrachain disulfides were present (Creighton, T., 1989). However, crystal protein run on reducing gels gives a protoxin band with the same mobility as the protoxin in the non-reducing gels in Figure 16, indicating that no intrachain disulfides are present. The results obtained therefore support the conclusion, drawn from the diagonal mapping, that all the disulfide linkages are interchain.

Competitive labelling

Competitive labelling (Kaplan, H. et al., 1971; Young, M. and Kaplan, H., 1989) of the solubilized protoxin was carried out at pH values between 7.0 and 10.0 with alanylalanine added as an internal standard. The solubilized protoxin from B. thuringiensis subsp. kurstaki HD-1 has a

molecular mass of 130 kDa and we have observed a tendency to form insoluble high molecular weight aggregates during the purification on Sephacryl S-300 columns. It also has a relatively low solubility at neutral pH values. The properties of the protoxin therefore make it necessary to study it in very dilute solution. Recent modifications in the competitive labelling methodology have made it possible to study proteins in very dilute solution (Hefford, M-A. et al., 1985). The present study was carried out at a protoxin concentration of 3.5×10^{-7} M where the protoxin is soluble over the entire pH range from 7 to 10.

No cysteine residues are present in the toxin, and therefore the reactivity of the sulfhydryl groups reflects properties of the C-terminal half of the protoxin molecule which is removed during the activation process. A non-linear least squares regression of the reactivity data gives a titration curve with a pK of 8.45 ± 0.13 and a reactivity (r) 39.6 ± 2.7 times that of the α-amino group of alanylalanine (Figure 17). The protoxin has 16-18 residues of cysteine per molecule and the fact that the average reactivity of these residues can be fitted to a regular titration curve with a small standard error in pK and reactivity indicates that the sulfhydryl groups in the solubilized protoxin have similar environments, and hence similar pK values and reactivities.

The average pK value obtained for the sulfhydryl groups is close to the pK value of approximately 8.5 observed for exposed thiols in model compounds and proteins (Shaked, Z. et al., 1980). This result along with the high reactivity observed strongly suggests that the majority if not all the sulfhydryl groups are fully exposed on the surface of the protoxin.

Usually sulfhydryl groups are found buried in proteins. The observation that all the sulfhydryl groups appear to be exposed in the protoxin is most unusual.In order to test this conclusion the native solubilized protoxin isolated from the B. thuringiensis subsp. kurstaki HD-1 crystal was reacted with iodoacetate and iodoacetamide in the absence of any denaturant. In the native protoxin, the sulfhydryl groups can be quantitatively derivatized with either reagent which is consistent with the conclusion that all the sulfhydryl groups are exposed on the surface of the protoxin.

We can conclude from this series of experiments that, in the protein crystal from B thuringiensis subsp kurstaki HD-1 and subsp. entomocidus, the cysteine residues form predominantly symmetrical interchain bridges. The sulfhydryl groups of the solubilized 130 kDa crystal protein (protoxin) are exposed on the surface of the molecule and the disulfides which are formed in the crystallization process are also

readily accessible. A mechanism which specifically orients the protein so that a regular disulfide-linked crystal lattice is formed must be involved. As there is evidence that cloned protoxin forms crystals in B. subtilis (Calogero, S. et al., 1989), a specific association of the protoxin molecules may be sufficient for the formation of the symmetrical interchain disulfide bridges. The exposed disulfides in the crystal protein make them susceptible to alkaline cleavage which appears to be important in the generation of toxin in the highly alkaline (pH 10-10.5) lepidopteran insect gut (Jaquet, F. et al., 1987).

Comparison of the gene nucleotide sequences of the known Bacillus thuringiensis genes (Höfte, H. and Whitely, H., 1989) shows that the cysteine containing regions of the protoxin are highly conserved. In families of proteins, where such conservation is observed, the precise structure is usually essential for biological activity. Höfte, H. and Whitely, H. (1989) have speculated that the structure in the C-terminal half is conserved because it is required for crystal formation. The present results which show that all the sulfhydryl groups are located on the surface of the protoxin and form symmetrical interchain bridges supports the hypothesis that a precise orientation of the sulfhydryl groups

is essential for crystal formation and ultimately its insecticidal activity.

Claims to original research

1) Methodology for the selective isolation of N-terminal peptide using HPLC.

2) A modification of the diagonal procedure of Brown and Hartley which makes its application to proteins of molecular mass greater than 40 kDa was developed.

3) Demonstration that contaminating proteinases are responsible for the degradation of the crystal protein upon solubilization.

4) The effect of solar irradiation on the tryptophan, histidine, methionine and tyrosine residues in the crystal protein was quantified. It was found that the destruction of these side-chains did not correlate with the loss in biological activity.

5) Quantification of the cysteine and cystine residues in crystal from two subspecies of B. thuringiensis.

6) Demonstration that the cysteine residues in the crystal protein form symmetrical interchain disulfide bridges.

REFERENCES

Adang, M., Stauer, M., Rocheleau, T., Leighton, J., Barker, R. and Thompson, D. (1985) Characterized Full-Length and Truncated Plasmid Clones of the Crystal Protein of Bacillus thuringiensis subsp. Kurstaki HD-73 and Their Toxicity to Manduca Sexta, Gene 36, 289-300.

Andrews, R.E.-Jr., Bibilos, M.M., Bulla, L.A.-Jr. (1985) Protease activation of the entomocidal protoxin of Bacillus thuringiensis subsp. kurstaki, Appl. Environ. Microbiol. 50, 737-742.

Andrews, R., Faust, R., Wabiko, H., Raymond, K. and Bulla, L. Jr. (1987). The biotechnology of Bacillus thuringiensis. CRC Crit. Rev. Biotechnol. 6, 163-230.

Angus, T.A. (1954). A bacterial toxin paralyzing silkworm larvae. Nature 172, 545.

Angus, T.A. (1956). Extraction, purification, and properties of Bacillus sotto toxin. Can. J. Microbiol. 2, 416-426.

Aronson, A., Beckman, W. and Dunn, P., (1986), Bacillus thuringiensis and Related Insect Pathogens, Microbiological Reviews 50, 1-24.

Auffray, Y., Boutibonnes, P., (1987), Photoreactivation Capacity of Ultraviole-Irradiated Bacillus thuringiensis. Mutation Research 192, 11-13.

Beegle, C., Dulmage, H., Wolfenbarger, D. and Martinez, E. (1981). Persistance of Bacillus thuringiensis var. kurstaki insecticidal activity on cotton foliage. Environ. Entomol. 10, 400-401.

Bennett, C., (1967) Paper Chromatography and Electrophoresis; Special Procedure for Peptide Mapping in Method of Enzymology, Vol. XI Ed. C.H.W. Hirs. p. 330-339.

Bietlot, H., Carey, P., Choma, C., Kaplan, H., Lessard, T. and Pozsgay, M. (1989). Facile preparation and characterization of the toxin from Bacillus thuringiensis var. kurstaki. Biochem. J. 260, 87-91.

Bietlot, H., Vishnubhatla, I., Carey, P., Pozsgay, M. and Kaplan, H. Characterization of the cysteine residues and disulfide linkages in the protein crystal of Bacillus thuringiensis. (In press).

Brizzard, B. and Whiteley, H. (1988). Nucleotide sequence fo an additional crystal Protein Gene from Bacillus thuringiensis. Nucleic Acids Res. 16, 2733-2724.

Brousseau, R. and Masson, L. (1988) Bacillus thuringiensis Insecticidal Crystal Toxins. Gene Structure and Mode of Action. Biotech. Adv. 6, 697-724.

Brown, J., Harteley, B., (1966), Localization of Disulfide Bridges by Diagonal Paper Electrophoresis, Biochem. J. 101, 214-228.

Bulla, L. Jr., Kramer, K. and Davidson, L. (1977). Characterization of the entomocidal parasporal crystal of Bacillus thuringiensis. J. of Bacteriol. 130, 375-383.

Calogero, S., Albertini, A.M., Fogher, C., Marzavi, R. and Galizzi, A. (1989), Appl. Environ. Microbiol. 55, 446-453.

Carey, P.R., Fast, P., Kaplan, H., Pozsgay, M., (1986) Molecular Structure of the Protein Crystal from Bacillus thuringiensis: A Raman Spectroscopic Study, Biochim. Biophys. Acta 872, 169-176.

Carlton, B. and Gonzalez, J. Jr. (1984). Plasmid-associated detal-endotoxin production in Bacillus thuringiensis in genetics and biotechnology of bacilli (A. Ganesan and J. Hoch eds.) pp. 387-400 Academic Press Inc. New York.

Chestukhina, G.G., Zalunin, I.A., Kostina, L.I., Kotova, T.S., Katrukha, S.P., Lyublinskaya, L.A., Stepanov, V.M. (1978) Proteinases bound to crystal of Bacillus thuringiensis Biokhimiya 43, 681-686.

Chestukhina, G.G., Kotova, T.S., Zalunin, I.A., Stepanov, V.M. (1979) Proteinases during growth and spore formation of Bacillus thuringiensis. Biokhimiya 44, 625-631.

Chestukhina, G., Zalvnin, J., Kostina, T., Kotova, T., Kattrukha, S. and Stepanov, V. (1980). Crystal-forming proteins of Bacillus thuringiensis. Biochem. J. 187, 457-465.

Couche, G.A., Pfannenstiel, M.A. and Nickerson, K.W. (1987) Structural Disulfide Bonds in the Bacillus thuringiensis subsp. israelensis Protein Crystal. J. of Bacteriol. 169, 3281-3288.

Creighton, T.E. (1989) In Proteins: Structure and Molecular Properties, p. 305-311, W.H. Freeman and Company, New York.

Darbre, A. (1986) Analytical Methods In Practical Protein Chemistry, A Handbook (A. Darbre (ed)) John Wiley & Son New York.

Dastidar, G. and Nickerson, K. (1979). Interchain crosslinks in the entomocidal Bacillus thuringiensis crystal protein. FEBS Lett. 108, 411-414.

Donovan, W., Dankogski, C., Gilbert, M., Gawron-Burke, M., Groat, R. and Carlton, B. (1988). Amino acid sequence and entomocidal activity of the P2 crystal protein. J. Biol. Chem. 263, 651-657.

Dulmage, H.T. (1978). Genetic manipulation of pathogens: Selection of different strains In Genetics in Relation to insect management (M.A. Hoy, J.J. McKelvey, Jr. ed.) pp. 116-127, Academic Press, New York.

Ellar, D., Thomas, W., Knowels, B., Ward, E., Todd, J., Drobniewski, F., Lewis, J., Sawyer, T., Last, D. and Nichols, C. (1985). Biochemistry, genetic and mode of action of Bacillus thuringiensis delta-endotoxins. In Molecular biology of microbial differentiation (J.A. Hoch and P.Setton eds.) pp. 230-240. ASM Washington, D.C.

Fast, P. and Donaghue, T. (1971). The delta-endotoxin of Bacillus thuringiensis II. On the mode of action. J. Invert. Pathol. 18, 135-138.

Fast, P.G. (1972). Delta-endotoxin of Bacillus thuringiensis III: A rapid method for separating parasporal bodies from spores. J. Invertebr. Pathol. 20, 139-140.

Fast, P., Murphy, D. and Sohi, S. (1978), Bacillus thuringiensis - Delta-Endotoxin: Evidence that Toxin acts at the Surface of Susceptable Cells, Experientia 34, 762-763.

Fast, P.G. and Martin, W.G. (1980) Bacillus thuringiensis crystal toxin: dissociation into toxic low molecular weight peptides. Biochem. Biophys Res. Commun. 95, 1314-1320.

Fast, P.G. (1981). The crystal toxin of Bacillus thuringiensis. In Microbial control of pest and plant discusses 1970-1980, (H.P. Burges (ed)) pp. 223-248, Academic Press Inc. New York.

Gharis, G. and Yon, J. (1982) in Protein Folding pp. 262-263, Academic Press, New York.

Grossweiner, L. (1976). Photochemical inactivations of enzymes. Current Topics in Radiation Research Quarterly 11, 141-199.

Grossweiner, L.I. (1984) Photochemistry of Proteins: A Review - Current Eye Res. 3, 137-144.

Hall, I.M. (1963) Microbial control, In Insect pathology, An Advance Treatise Vol. 2 Steinhous, E.A. ed. Academic Press, New York pp. 477.

Hara, S., Makino, J. and Ikewaka, T. (1989) Amino Acid Sequences and Disulfide Bridges of Serine Proteinase Inhibitors from Bitter Gourd (Momordica charantia Linn) Seeds, J. Biochem. 105, 88-92.

Harms, R.L., Martinez, D. and Griego, V., (1986), Isolation and Characterization of Coproporphyrin Produced by Four Subsepcies of Bacillus thuringiensis, Appl. and Environ. Microbiol. 51, 481-486.

Hefford, M.-A., Evans, R.M., Oda, G. and Kaplan, H. (1985), Unusual Chemical Properties of N-Terminal Histidine Residues of Glucagon and Vasoactive Intestinal Peptide. Biochemistry 24, 867-874.

Heimpel, A. and Angus, T. (1963) Diseases caused by certain sporeforming bacteria, In Insect Pathology, an Advance Theatise. Vol. 2 Steinhous, E.A. ed. Academic Press, New York pp. 21.

Himeno, M. (1987), Mechanism of Action of Delta-Endotoxin from Bacillus Thuringiensis, J. Toxicol. - Toxin Reviews 6, 45-71.

Himeno, M., Koyama, N., Furato, J. and Komano, T. (1987). Mechanism of action of Bacillus thuringiensis insecticidal delta-endotoxin on insect cells in vitro. Agric. Biol. Chem. 49, 1461-1468.

Hirs, C.H.W. (1956). The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219, 611-621.

Hofte, H. and Whiteley, H. (1989). Insecticidal crystal proteins of Bacillus thuringiensis, Microbiol. Rev. 53, 242-255.

Honée, G., van der Salm, T. and Visser, B. Nucleotide sequence of crystal protein gene isolated form B. thuringiensis subspecies entomocidus 60.5 coding for a toxin highly active against spodoptera species. Nucleic Acid Res. 16, 6240.

Huber, E.H., Lüthy, P. (1981) In Pathogenisis of Invertebrate Microbial Diseases (E.W. Davidson, ed) pg. 209-228, Allenheld Osmun, Totowa, N.J.

Huber, H.E., Luthy, P., Ebersold, H.R. and Cordier, S.L. (1981) The Subunits of the Parasporal crystal of Bacillus thuringiensis: Size, Linkage, and Toxicity. Arch. Microbiol. 129, 14-18.

Ishiwata, S. (1901). On a kind of severe Flacherie (sottodisease) Dainihon Sanshi Keiho 9, 1-5.

Jaquet, F., Hütter, R. and Lüthy, P. (1987). Specificity of Bacillus thuringiensis delta-endotoxin. Appl. Environ. Microbiol. 53, 500-504.

Jensen, P. and Sottrup-Jensen, L. (1986). Primary Structure of Human α_2-macroglobulin - complete Disulfide Bridge Assignment and Localization of two Interchain Bridges in the Dimeric Proteinase Binding Unit. J. Biol. Chem. 261, 15863-15869.

Kaplan, H., Stevenson, K. and Hartley, B. (1971). Competitive labelling a method for determining the reactivity of individual groups in proteins. Biochem. J. 124, 289-299.

Kaplan, H., Long, B. and Young, M. (1980) Chemical Properties of Functional Groups of Mouse Immunoglobulins of the IgA, IgG2a and FgM Classes. Biochemistry 19, 2821-2827.

Kaplan, H., Oda, G., Bietlot, H., Carey, P., Pozsgay, M. and Fast, P., (1986). Selective Isolation of Free or Blocked Amino-Terminal Peptides from Digest by High Performance Liquid Chromatography: Application to the Crystal Protein of Bacillus thuringiensis, Biochem. Cell Biol. 64, 1226-1233.

Knowles, B. and Ellar, D., (1987) Colloid-Osmotic Lysis is a General Feature of the Mechanism of Action of Bacillus thuringiensis, Delta-Endotoxin with Different insect Specficity, Biochim. Biophys. Acta 924, 509-518.

Kondo, S., Tamvra, N., Konitate, A., Hattori, M., Akashi, A. and Ohmori, I. (1987). Cloning and nucleotide sequencing of two insecticidal delta-endotoxin genes from Bacillus thuringiensis var. kurstaki HD-1 DNA. Agric. Biol. Chem. 51, 455-463.

Kronstad, J. and Whiteley, H. (1986). Three classes of homologous Bacillus thuringiensis crystal protein genes. Gene 43, 29-40.

Means, G. and Feeny, R. (1972) In Chemical Modification of Protein. Holden-Day Inc. London p. 160.

Minnich, S. and Aronson, A. (1984) Regulation of Protoxin Synthesis in Bacillus thuringiensis, J. of Bacteriol. 158, 447-454.

Morris, O.N. (1983). Protection of Bacillus thuringiensis from inactivation by sunlight. Can. Ent. 115, 1215-1227

Nakagawa, M. (1980). Oxygenation of Tryptophan to formyl-kunurenine-dye-sensitized photooxygenation in Biochemical and Medical Aspects of Tryptophan Metabolism (O. Hayaishi, Y. Ishimura and R. Kido eds.). pp. 49-58. Elsevier/North Holand Biomedical Press Amsterdam.

Nickerson, K.W. (1980) Structure and Function of the Bacillus thuringiensis protein crystal. Biotechnol. Bioeng. 12, 1305-1335.

Norris, O. (1971). The protein crystal toxin of Bacillus thuringiensis: Biosynthesis and physical structures. In Microbial control of insects and mites (H. Burges and N. Hussey eds.) pp. 229-246, Academic Press, London

Perham, R. (1967) A Diagonal Paper Electrophoretic Technique for Studying Amino Acid Sequence Around Cysteine and Cystine Residues of Proteins. Biochem. J. 105, 1203-1207.

Pileni, M-P., Santus, R. and Land, E. (1978). On the photosensitizing properties of N-formylkynurenine and related compounds. Photochem. Photobiol. 28, 525-529.

Pozsgay, M., Fast, P., Kaplan, H., Carey, P.R., (1987). The Effect of Sunlight of the Protein Crystals from Bacillus thuringiensis var. Kurstaki HD-1 and NRD-12: a Raman Spectroscopic Study, J. of Invert. Path. 50, 246-253.

Préfontaine, G., Fast, P., Lau, P., Hefford, M., Hanna, Z., Brousseau, R. (1987), Use of Oligonucleotide Probes to Study the relatedness of Delta-Endotoxin Genes Among Bacillus thuringiensis Subspecies and Strains, Appl. and Environ. Microbiol. 53, 2808-2814.

Rice, R. and Means, G. (1971), J. Biol. Chem. 246, 831-832.

Riechelderfer, C. (1985) Biological assays with insect pathogens. In Handbook of Natural pesticides: Methods Vol. I. (N. Bhush and Mandave eds). CRC Press pp. 493.

Rusco, C. (1987) The attitude and role of the agrochemical industry towards pesticide resistance. In Combating Resistance to Xenobiotics: Biochemical and Chemical Approaches. (Ford, M., Holloman, D., Khambag, B. and Sawicki, R. eds.) Ellis Horwood Publishers pp. 26-31. New York.

Sandberg, S., Romslo, I., Houding, G. and Bjorndal, T. (1982), Porphyrin - Induced Photodamage as Related to Subscellular Localization of the Porphyrins. Acta Dermatol. Suppl. 100, 75-80.

Sattele, D., Haniff, C., Thomas, W. and Ellar, D. (1985). Laser-diffusion measurments on δ-endotoxin crystals from Bacillus thuringiensis var. kurstaki HD-1. Biochimica et Biophysica Acta 840, 423-427.

Sayles, V., Aronson, J. and Rosenthal, A., (1970) Small Polypeptide Components of the Bacillus thuringiensis Parasporal Crystalline Inclusion, Biochem. Biophys. Res. Comm. 41, 1126-1133

Schnepf, E., Wong, H. and Whitley, H. (1985) The Amino Acid Sequence of a Crystal Protein From Bacillus thuringiensis Deduced from the DNA Base Sequence, J. Biol. Chem. 260, 6264-6272.

Shaked, Z., Szajewski, P. and Whitesides, G. (1980). Rates of thiol-disulfide interchange reactions involving proteins and kinetic measurements of thiol pKa values. Biochemistry 19, 4156-4166.

Shaltiel, S. (1967), Thiolysis of Some Dinitrophenyl Derivatives of amino acids. Biochem. Biophys. Res. Comm. 29, 178-183.

Shibano, Y., Yamagata, A., Nakamura, N., Iizuka, T., Sugisaki, H. and Takanami, M. (1985). Nucleotide sequence coding for the insecticidal fragment of the Bacillus thuringiensis crystal protein. Gene 34, 243-251.

Silva, C. and Cidlowski, J. (1989) Direct Evidence for Intra- and Intermolecular Disulfide Bond Formation in the Human Glucocortious Receptor. J. Biol. Chem. 264, 6638-6647

Simpson, R., Neuberger, M. and Liu, T. (1976). Complete amino-acid analysis of proteins from a single hydrolysate. J. Biol. Chem. 251, 1936-1940.

Sottrup-Jensen, L., Lonblad, P., Jones, C. and Stepanik, T., (1984). Primary Structure of Eight CNBr Fragments Located in the NH_2-Terminal Half of α_2-macroglobulin, Accounting for 603 Amino Acid Residues. J. Biol. Chem. 259, 8304-8309.

Sottrup-Jensen, L., Stepanik, T., Jones, C., Lonblad, P. Kristensen, T. and Wieizbicki, D. (1984). Primary Structure of Human α_2-macroglobulin-isolation of the 26 CNBr Fragments, Amino Acid Sequence of 13 Small CNBr Fragments, Amino Acid Sequence of Methionine Containing Peptides and Alignment of all CNBr Fragments. J. Biol. Chem. 259, 8293-8303.

Statistic Canada (1987) Selected Forestry Statistics Canada: Information Report Ex40.

Sternhaus, E.A. (1951), Possible use of Bacillus thuringiensis Berliner as an aid in the biological control of the alfalfa caterpillar, Hilgardia 20, 359.

Visser, B., van der Salm, T., van der Brink, W. and Fakers, G. (1988). Gene from Bacillus thuringiensis entomocidus 60.5 coding for insect-specific crystals proteins. Mol. Gen. Genet. 22, 219-224.

Volkin, D. and Klibaunow, A. (1988) Minimizing Protein Inactivation In Protein Function: A Practical Approach. T.E. Creighton (ed) CRL Press, New York.

Wessling-Resnick, M. and Johnson, G. (1989) Evidence for Oligomeric Forms of Transducing Alpa Subunits: Formation of Intermolecular Alpha Disulfide Linkages. Biochem. Biophys. Res. Comm. 159, 651-667.

Whiteley, H.R. and Schnepf, E.H., (1986), The Molecular Biology of Parasporal Crystal Body Formation in Bacillus thuringiensis, Ann. Rev. Microbiol. 40, 549-76.

Yamamoto, T. and McLaughlin, R. (1981). Isolation of a protein from the parasporal crystal of Bacillus thuringiensis var. kurstaki toxic to the mosquito larvae Aedes tueniorhynchus. Biochem. Biophys. Res. Commun. 103, 414-421.

Yamamoto, T., Ehmann, A., Gozalez, J. Jr. and Carlton, B. (1988). Expression of three genes coding for 135-kDa endomocidal protein in Bacillus thuringiensis var. kurstaki. Current Microbiol. 17, 5-12.

Young, M. and Kaplan, (1989). IN Protein structure and function: A Practical Approach (T.E. Creighton, ed) pp. 225-244 CRC Press, Oxford.